EFFECTS OF PLOIDY LEVEL ON THE REPRODUCTIVE BIOLOGY OF TROPICAL *ACACIA* SPECIES

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ABSTRACT

Acacia mangium Willd., *A. auriculiformis* A. Cunn. ex Benth and their natural hybrid, *A. mangium* x *A. auriculiformis*, are important plantation species for timber production in the tropics. However, their potential to invade natural ecosystems has been a concern because of their prolific production of long-lived seeds. Deployment of triploid *Acacia* clones in plantations might reduce fertility and associated weed risk.

Studies were conducted in a hybridizing orchard that was established in southern Vietnam in 2003 with alternate rows of diploid *A. mangium* (AM-2*x*), diploid *A. auriculiformis* (AA-2*x*) and colchicine-induced autotetraploid *A. mangium* (AM-4*x*) clones. Heavy flowering and seed production was obtained for all three species/ploidy combinations; however, the yield of viable open-pollinated triploid (3*x*) seeds from open pollinated seed had been very low. The reproductive behaviour of the three species/ploidy combinations in the orchard was therefore investigated to determine whether there are barriers to the production of triploid progeny within and between these two species.

Peak flowering period for both AM-2*x* and AM-4*x* (November – December) was slightly earlier than for AA-2*x* (December – January). The spikes of AM-4*x* were shorter than those of AM-2*x*, resulting in fewer flowers per spike, but they were longer and had more flowers than AA-2*x*. The proportion of male to hermaphrodite flowers was similar for all three species/ploidy combinations. AM-4*x* flowers had shorter styles, but the stigma and polyad diameters were greater than those of AM-2*x*. Differences in stigma and polyad size between cytotypes were not

sufficient to adversely affect inter-cytotype pollination. Pollen recovered from the bodies of the main insect pollinators (honeybees) indicated that they did not discriminate in their foraging behaviour. Therefore, neither the floral phenology and morphology of species/ploidy categories nor the pollinator foraging behaviour created barriers to inter-cytotype pollination.

Pollen-pistil interactions following different mating combinations within and between each of AM-4*x*, AM-2*x*, and AA-2*x* were investigated. Following controlled pollinations, AM-4*x* ovules exhibited more attraction to self- than outcross- pollen tubes, in contrast to AM-2*x* and AA-2*x*; however, this trend was not consistent for all of the genotypes examined. The reciprocal crosses of AA-2*x* and AM-2*x* were successful as pollen tubes grew well in both AM-2*x* and AA-2*x* styles and penetrated their ovules. For inter-cytotype crosses, inter-species, particularly those with AA-2*x* as the maternal parent, had a significantly greater number of ovules penetrated than did intra-species crosses. However, yields of pods and filled seeds following all inter-cytotype crosses were extremely poor, compared to those from the intra-cytotype crosses. Thus, there were strong barriers to production of viable 3*x* progeny, despite the demonstrated absence of pre-zygotic isolation.

Ovule abortion occurred more frequently in all inter-cytotype crosses than in the open-pollinated flowers at 5 and 7 weeks after pollination. Consequently, the proportion of filled seeds set per pod for inter-cytotype crosses was far lower than in pods arising from intra-cytotype crosses and open-pollination. Moreover, the weight of filled seeds from inter-cytotype crosses was significantly lighter than filled seeds from open-pollination, and they were unable to germinate. Analysis using

microsatellite markers of DNA obtained from these non-germinated seeds confirmed that most were triploid and had resulted from the target inter-cytotype crosses.

It was concluded that abnormal seed development and abortion occurred throughout the 18-week period of pod development, resulting in failure of this set of inter-cytotype crosses to produce any viable triploid progeny. Possible reasons for this are discussed. Research on application of *in vitro* culture techniques to immature embryos may be required to recover triploid progeny from inter-cytotype crosses of these *Acacia* species, as has been achieved for other species.

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CHAPTER 1. INTRODUCTION

This chapter introduces the *Acacia* species studied, including their natural distribution, introduction and utilization history, and some aspects of reproductive biology that might be involved in invasiveness. The importance of polyploidy in *Acacia* and aims of the study are also indicated.

1.1. The genus *Acacia*

The genus *Acacia* is the second largest in the family *Leguminosae*, distributed throughout the tropical and warm temperate areas of the world. It was first described by Philip Miller in 1754 and subsequently a number of studies have been undertaken to reassess the taxonomic and phylogenetic status of this large genus (MASLIN 2011; THIELE *et al.* 2011). The classification was widely adopted viewing *Acacia* Miller as a cosmopolitan genus containing about 1,350 species in three subgenera, namely *Acacia*, *Senegalia* (syn. *Acacia* subg. *Aculeiferum*) and *Racosperma* (syn. *Acacia* subg. *Phyllodineae*) (PEDLEY 1986). Other current molecular and phylogenetic evidence suggests that the genus *Acacia* can be split into at least six major monophyletic lineages, including subg. *Acacia*, subg. *Aculeiferum*, subg. *Phyllodineae*, sect. *Filicinae*, sect. *Spiciflorae* and the "*Acacia coulteri*" group (MASLIN *et al.* 2003a; MASLIN *et al.* 2003b; MILLER *et al.* 2003; MURPHY *et al.* 2000; ROBINSON and HARRIS 2000; THIELE *et al.* 2011). More recently, the Australian wattles (formerly *Acacia* subg. *Phyllodineae*, now *Acacia* subg. *Acacia*) would be called the name *Acacia* (SMITH and FIGUEIREDO 2011). The alternative classification can, however, be applied.

Subgenus *Phyllodineae* is the largest monophyletic group within the genus *Acacia*, with more than 1022 species, of which only 12 species occur outside the Australian continent (MASLIN 2011). These Australian acacias are present in almost all major terrestrial habitats of Australia, particularly in arid and semi-arid areas, and in neighbouring Indonesia, Papua New Guinea (PNG) and some other Pacific island nations (MIDGLEY and TURNBULL 2003). *Acacia mangium* Willd. and *A. auriculiformis* A. Cunn. ex Benth. belong to sect. *Juliforae*, one of 7 sections of the subgenus *Phyllodineae*, have been called "*Acacia* Wood" or simply "*Acacia*" in international trades since 2005 (GRIFFIN *et al.* 2011).

Together with eucalypts, acacias dominate Australia's vegetation, particularly on sites with very nutrient-poor soil (GROVES 1994). Many *Acacia* species are not heavily exploited, being grown only for amenity and land amelioration (MASLIN 2002). Some Australian *Acacia* species have been extensively planted outside Australia for various purposes, including land stabilization, reforestation and multi-purpose plantations (MIDGLEY and TURNBULL 2003; THOMSON *et al.* 1994). Plantations of tropical species including *A. mangium*, *A. auriculiformis,* and more recently *A. crassicarpa* A.Cunn ex Benth. Are particularly important (GRIFFIN *et al.* 2011; MASLIN 2002; MIDGLEY and TURNBULL 2003).

Acacia mangium and *A. auriculiformis* are tropical tree species known for their rapid growth and multiple uses. Their wood is suitable for a range of uses, such as sawn timber, pulp and fuel wood, and they are also planted for erosion control and land rehabilitation. They are adapted to a variety of tropical humid environments including acidic and nutrient-deficient soils (MIDGLEY and TURNBULL 2003). These

characteristics have made them promising species for forest plantation in many countries where degraded land and/or diverse climatic regimes are present.

1.2. The importance of *Acacia mangium*, *A. auriculiformis*, and *Acacia* hybrid

1.2.1. *Acacia mangium* Willd.

Acacia mangium Willd. is native to Australia, PNG, and Indonesia. Its natural distribution extends from northeast Queensland in Australia through the Western Province of PNG to the islands of Sula, Ceram and Aru in Indonesia, between 1^{o} - $18^{o}57'$ S latitude and $125^{o}22'$ - $146^{o}17'$ E longitude. It is typically a low-elevation species, usually occurring at altitudes below 300 m (AWANG and TAYLOR 1993) (Fig. 1.1). *Acacia mangium* is large tree, reaching up to 30 m in height and prefers humid climates with an annual rainfall of 1,000 - 4,500 mm. It shows vigorous growth on suitable tropical sites, with 3 - 4 cm increase in stem diameter per year and annual wood volume increment of up to 30 m^3 ha^{-1} (MIDGLEY and TURNBULL 2003).

The basic physical and chemical wood properties of *A. mangium* have been well studied (AWANG and TAYLOR 1993). In general, *A. mangium* wood has moderate fiber morphology (*e.g.* fiber length, width, lumen and wall thickness) and cellulose content and pulp yield are slightly lower than average temperate hardwood; however, it is still favoured for pulping. Besides, *A. mangium* wood has dense and straight grained as well as a distinction between sapwood and heartwood with light cream to dark brown in colour. The wood is suitable for processing some commercial products, such as furniture, cabinets, moldings, and door/ window components.

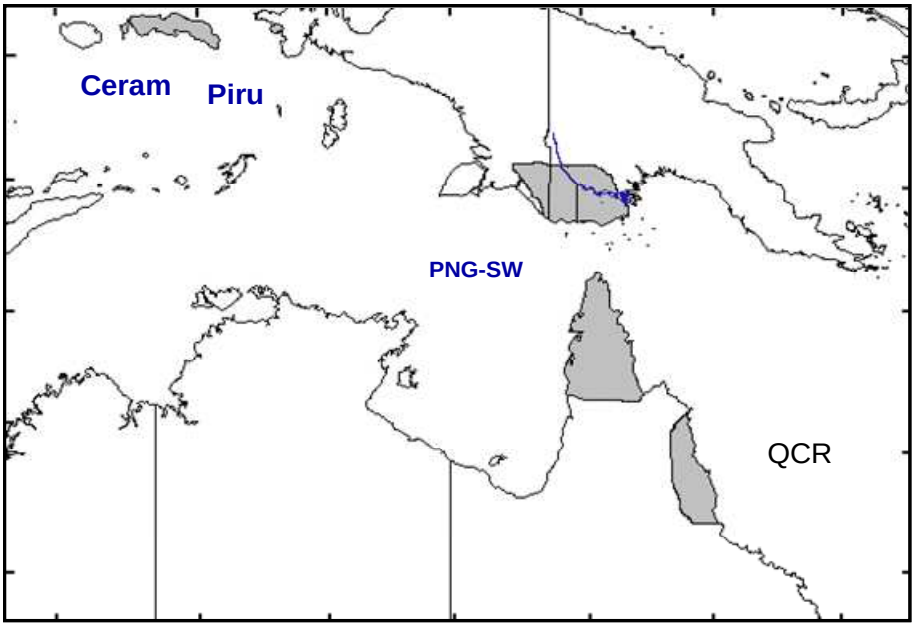


Figure 1.1. Natural distribution of *A. mangium* (AWANG and TAYLOR 1993)

1.2.2. *Acacia auriculiformis* A. Cunn. ex Benth.

Acacia auriculiformis A. Cunn. ex Benth. also grows naturally in Australia, PNG and Indonesia between latitudes 9 – 16°S and longitudes 130 – 145°E and altitudes of up to 400 m above sea level (WICKNESWARI and NORWATI 1993) (Fig. 1.2). *Acacia auriculiformis* can attain up to 30 m in height and 80 cm in diameter with a straight, single stem on favorable sites (BOLAND *et al.* 1990).

The wood is heavy and straight grained, with high percentage of heartwood that is light brown or dark red in colour (JAHAN *et al.* 2008). In addition, the wood is easy to polish and finishs well with sharp tools. Hence, *A. auriculiformis* wood is suitable for sawn timber applications, such as furniture-making, flooring and structural timber. Its wood properties (*e.g.* wood basic density, stiffness, shrinkage, and strength) can be improved by breeding programs (HAI 2009; HARWOOD *et al.* 2008).

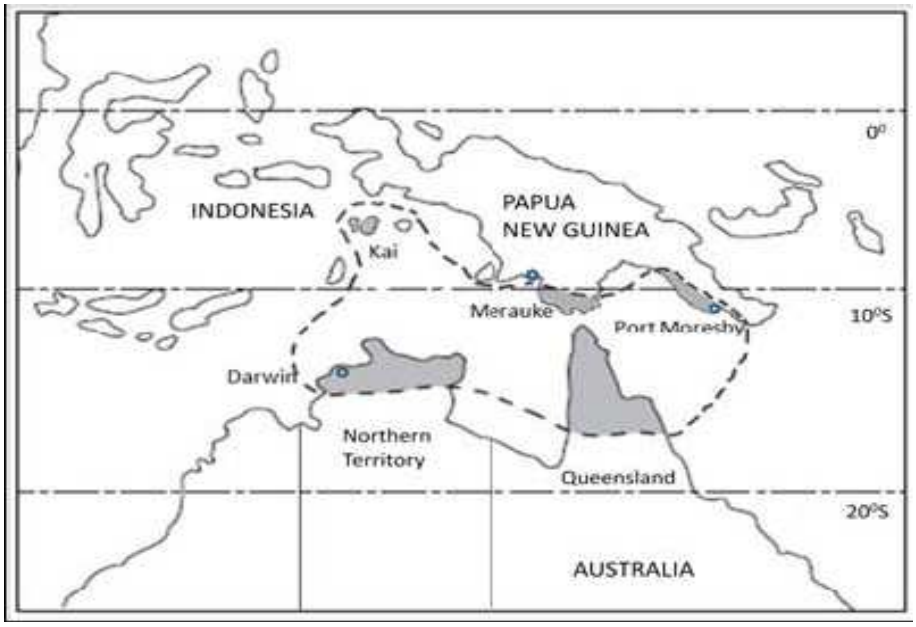


Figure 1.2. Natural distribution of *A. auriculiformis* (PINYOPUSARERK 1990)

1.2.3. Natural *Acacia* hybrid (*A. mangium* x *A. auriculiformis*)

Natural inter-specific hybrid (*A. mangium* x *A. auriculiformis*) (referred to as "*Acacia* hybrid") individuals were first reported in *A. mangium* plantations planted in Sabah, Malaysia in 1972 and subsequently observed in several countries such as Australia, China, Indonesia, Malaysia, Thailand and Vietnam (CARRON and AKEN 1992; TURNBULL *et al.* 1998). A number of studies on growth and wood properties for pulp yield indicated the potential to capture hybrid vigour of *Acacia* hybrid (CARRON and AKEN 1992; KHA 2000; KHA 2001; TURNBULL *et al.* 1998). It should be noted that the growth and tree form of F_2 hybrid seedlings (*e.g.* seeds collected from F_1 hybrid trees) displayed obvious segregation of parental phenotypes (KHA *et al.* 1996; METCALFE and WOO 1998). Therefore, outstanding F_1 clones have been selected and mass-propagated for short-rotation plantations in Malaysia and Vietnam.

In Vietnam, natural *Acacia* hybrid presented higher growth rate, greater resistance to disease, better adaptability to different soil types, and heterosis in some wood properties than the pure parental species parents (KHA 2000; KHA 2001; KHA and PHUC 1995). The mean growth rate of the hybrid was almost double (22 m^3 ha^{-1} yr^{-1}) compared with that of the pure parents (12 m^3 ha^{-1} yr^{-1}) and the pulping productivity was 232 kg pulp m^{-3} of wood volume, higher than 195 kg m^{-3} for *A. mangium*. Besides, its timber properties were reported to be intermediate to those of *A. mangium* and *A. auriculiformi*, making it valuable for the paper-making and particleboard or/and medium-density fibreboard production (Kha 2001).

1.2.4. History of introduction and utilization

Tropical *Acacia* species have been introduced into Asia, especially Southeast Asian countries, in the late years of the 19th century onwards. As reviewed by MIDGLEY and TURNBULL (2003), *A. auriculiformis* was first introduced to Malaysia in 1932, Thailand in 1935 and Vietnam in the 1960s, while *A. mangium* was planted in Malaysia in 1966 and Vietnam in the early 1980s (NGHIA and KHA 1998). Recently, natural *Acacia* hybrid have been planted widely in Vietnam (KHA 2001).

Most of the large-scale plantations of *A. mangium* in Malaysia and Indonesia were to produce wood for pulp production or medium density fiberboard and only a small proportion of plantations were established for producing larger logs for plywood (MIDGLEY and TURNBULL 2003; TURNBULL *et al.* 1998). *Acacia auriculiformis* had been widely planted for afforestation programs and soil and water conservation in upland China since the 1970s (ZENG and YANG 1994) and reforestation in the southern Indian states, especially on coastal sands, waterlogged soils and industrial wasteland (*e.g.* coal mine spoil and paper mill sludge)

(KUSHALAPA 1991). Both *A. auriculiformis* and *A. mangium* have been planted intensively to provide material for wood production in Philippines (MIDGLEY *et al.* 1996). TURNBULL *et al.* (1998) reported that the planting area of these tropical *Acacia* species had steadily increased throughout Asia.

In Vietnam *A. mangium*, *A. auriculiformis*, and their hybrid are considered as the main species for short-rotation wood production plantations. The planting areas of these species increased rapidly with annual expansion rates of 10,000 to 15,000 ha per year. As a result, there were over 154,000 hectares of *A. mangium*, 92,000 hectares of *A. auriculiformis* and over 250,000 ha clonal plantations of *Acacia* hybrid plantations in Vietnam by 2009 (Ministry of Agriculture and Rural Development - MARD 2009, unpublished data). Plantations growing these *Acacia* species have partially satisfied the requirements for industrial wood, such as producing pulp and paper on the domestic market, exporting wood chip to foreign markets, and processing higher value end-uses (*e.g.* sawlogs with standard size of small-end diameter of at least 15 cm which are suitable for furniture, framing and flooring) in recent years (GRIFFIN *et al.* 2011; HAI 2009; HARWOOD *et al.* 2008).

1.3. Breeding programs for tropical Australian *Acacia* species in Vietnam

In the period from 1986 to 1992, five tropical Australian *Acacia* species, namely *A. mangium, A. auriculiformis, A. crassicarpa, A. aulacocarpa,* and *A. cincinnata* played an important role in forest planting strategies to supply raw material and environmental protection in Vietnam (KHA 2003; NGHIA 1996; NGHIA and KHA 1998). Provenance trials of these species were established in several parts of Vietnam. The provenances that performed outstandingly for both growth rate and

adaptability, are Coen River, Kings Plains, Wenlock River, Halroyed (Qld), Mibini and Morehead (PNG) for *A. auriculiformis* and Cardwell, Pongaki, Gubam, Iron Range, Pascoe River (Qld) and Derideri (PNG) for *A. mangium* (NGHIA and KHA 1998).

Species/provenance trials were converted to seed production areas (SPA) to supply improved seeds. Seedling seed orchards (SSO) and clonal seed orchards (CSO) then followed with a broad genetic base to produce genetically improved seeds for production forestry and led to significant improvements in yield and quality of acacias plantations in Vietnam as well as domestic and overseas seed exchanges in recent years (KHA 2003).

For long-term breeding, several progeny tests and clonal tests of *A. auriculiformis* and *A. mangium* were established to develop a comprehensive genetic base population for breeding. Knowledge of genetic parameters, genotype by environment interactions (G x E), and predicted gains, realized gains for traits of interests, which are related to growth performance and wood properties, was addressed for an intensive breeding program. Such comprehensive research on *A. auriculiformis* was investigated by HAI (2009). A similar study on *A. mangium* is now under way.

A program of selection and propagation for first generation *Acacia* hybrid was commenced in 1992. A series of clonal trials at multiple locations were established (KHA *et al.* 1996) and nineteen outstanding individual clones were then approved by MARD for use in production plantations.

Rapid expansion in short-rotation plantations of such species could however result in the possibility of invasion of native forest and cultivated areas. This concern has been raised in the recommendations of an international acacia workshop (TURNBULL *et al.* 1998) and numerous recent reviews (GRIFFIN *et al.* 2011; MILLER *et al.* 2011; RICHARDSON *et al.* 2011; WILSON *et al.* 2011).

1.4. Some aspects of reproductive biology of *Acacia* species

1.4.1. Phenology

Phenology refers to periodic biological phenomena, such as flowering, fruit/pod setting and dispersal, in relation to certain climatic conditions. For breeding programs, investigation of phenology, particularly information on synchrony in flowering time, is an important factor for success of both seed orchards and manipulated hybridization (SEDGLEY 1996; SEDGLEY and GRIFFIN 1989).

The reproductive phenology characteristic of a species is modified by the growing conditions (MILTON and MOLL 1982). For *A. mangium* and *A. auriculiformis*, differences in geographical location (*e.g.* latitude, longitude and climate regimes) resulted in differences to their peak flowering time when trees growing in Queensland, Australia and Malaysia were compared (SEDGLEY *et al.* 1992d). Therefore, the strategy for forest tree breeding should include information on floral phenology of the studied species in relevant locations.

1.4.2. Flower morphology and biology

1.4.2.1. Flower morphology

Acacias are andromonoecious plants with a compound raceme, called an inflorescence or a spike, that is either globose or spicate, bearing small hermaphrodite and staminate flowers (KENRICK 2003). The proportion of hermaphrodite to male flowers within the inflorescence differs dramatically between species and at different flowering densities. TYBIRK (1993) reported that *A. senegal* has only hermaphrodite flowers, while *A. tortilis* and *A. nilotica* have both hermaphrodite and male flowers. The proportion of staminate flowers in *A. mearnsii* was 52 % in light flowering seasons, compared to 93 % in heavy flowering seasons (MONCUR *et al.* 1991).

Acacia inflorescences are arranged singly, paired, several in the upper axils or in racemes or panicles of heads. Individual flowers within an inflorescence are similar in size and shape but differ in colour, with cream-white or bright-yellow flowers. The flower has a calyx 0.6 - 0.8 mm long with short obtuse lobes and the corolla is twice as long as the calyx (ORCHARD and WILSON 2001). Each flower has a single pistil and a large number of stamens (*e.g.* 34 in *A. pycnantha* and 537 in *A. myrtifolis*). However, acacias are all dichogamous and almost all Australian acacias are protogynous, the receptivity of the stigma preceding pollen shedding, whilst some African species (*e.g. A. nilotica, A. senegal* and *A. albida*) are protandrous, with pollen shed prior to stigma receptivity (SEDGLEY and HARBARD 1993).

1.4.2.2. Male reproductive biology

The anther of *Acacia* is bilobed and set terminally on the filament. Each lobe has four separate loculi paired one above the other, making eight loculi per anther in total. Inside each loculus is a culture sac that is formed by spherical orbicules joined together (KENRICK 2003; KENRICK and KNOX 1979).

The anther is broken vertically at an area of thin-walled cells to release the polyads out of the orbicule-lined sac when the surrounding tissue dries (KENRICK 2003; KENRICK and KNOX 1979; SORNSATHAPORNKUL and OWENS 1998b). STONE *et al.* (1998) indicated differences in daily timing of flower opening and pollen releasing in numerous African acacias, *e.g.* flowers of *A. nilotica* and *A. tortilis* both open well before dawn and release their pollen at or shortly after sunrise while *A. senegal* flowers open in mid- or late-morning and anther dehiscence follows immediately. Such temporal variation in pollen release among co-flowering species could reduce competition for pollination through pollinator visits.

The *Acacia* polyad, a compound pollen unit, exhibits polymorphism for pollen grain number (*e.g.* four, eight, 12 and 16 grains in Australian species, and 16 and 32 grains in African species) (KENRICK 2003; KENRICK and KNOX 1982). The number of grains per polyad depends on the number of pollen mother cells undergoing meiosis within each loculus (SEIJO and NEFFA 2004). The polyad of *A. mangium* and *A. auriculiformis* consist of 16 pollen grains arranged in the anther (NGAMKAJORNWIWAT and LUANGVIRIYASAENG 1991; SORNSATHAPORNKUL and OWENS 1998b). The 16 grain-polyad is a result of two cell duplications by mitosis each leading to four genetically identical daughter cells (KENRICK and KNOX 1982). KENRICK (2003) judged that the polyad represents all the products of meiosis and the

members of each tetrad can be identified and considered as the product of a single spore sac. Anthers are bilobed, each lobe consists of four loculi, each bearing only one polyad. Hence, the total number of pollen grains per anther is 128.

Acacia pollen viability was explored by SEDGLEY and HARBARD (1993). Storage of pollen for hybridization when parent trees flower asynchronously and are separated geographically has been evaluated. Freeze drying and liquid nitrogen are the most successful methods for long term pollen storage, although reduction in pollen viability varies between species (SEDGLEY 1989a; SEDGLEY and HARBARD 1993). Pollen viability experiments on *Acacia*, including pollen staining and *in vitro* germination methods showed that both *in vitro* pollen tube growth and TTC (2, 3, 5-triphenyltetrazolium chloride) or X-Gal (5-bromo-4-chloro-3-indole-β-galactoside) have not been reliable indicators because they did not present a true assessment of pollen fertility. Eventually, FDA (fluorescein diacetate) was found to give the best approximation of pollen viability although it required staining techniques (SEDGLEY and HARBARD 1993).

1.4.2.3. Female reproductive biology

In most *Acacia* species, the style is a narrow rod-shaped structure with a shallow cup-shaped and wet, non-papillate stigma type (KENRICK and KNOX 1981b). The size of stigma cup is always large enough to accommodate a single polyad. Style length varies depending on species or even populations of the same species (*e.g. A. terminalis* style length is shorter in early-flowering populations) (KENRICK 2003). During the flower opening stage, the living stigma produces a moist exudate which facilitates pollen adhesion (KENRICK and KNOX 1981a). The amount of exudate depends on the maturity of the stigmas (MARGINSON *et al.* 1985). Cytochemically,

the exudate contains proteins, carbohydrates, and spherical globules of lipids, but polyphenols are absent (KENRICK and KNOX 1981a).

In receptive styles, the exudate on the stigma persists for some hours and no exudate is present on non-pollinated or damaged stigmas. The exudation is considered to ensure attraction of pollinators for pollen transfer as well as pollen hydration and germination, pollen tube growth and direction (KENRICK 1994; KENRICK 2003; KENRICK and KNOX 1981a).

The ovary of *Acacia* species is pod-shaped and hairy containing 5 - 15 ovules. Ovules are attached in a double row by a long funicle to the placenta. The ovule is a structure that is specialized to form the seed after fertilization (BRUKHIN *et al.* 2005). It contains the embryo sac enclosed in the nucellus with two rudimentary integuments that cover only half of the embryo sac, resulting in the ovules becoming anatropous after fertilization which means the ovule is inverted and the micropyle is adjoined to the funiculus (KENRICK 2003; NGAMKAJORNWIWAT and LUANGVIRIYASAENG 1991; SORNSATHAPORNKUL and OWENS 1999a; SORNSATHAPORNKUL and OWENS 1999b).

1.4.3. Pollination ecology

Acacia pollen, together with the anther gland and floral fragrance, is offered as reward for pollinator visits. The movement of pollen would be either within a tree by gravity or between trees by visitors, mainly insects and birds (BERNHARDT 1989) because the size of *Acacia* polyads, ranging from 23 to 64 µm in diameter without air sacs, is not suitable for effective wind transport (KENRICK 2003). Therefore, most *Acacia* species require pollinators to transfer the pollen from the anther to the stigma

for reproductive function; this is particularly important in the context of seed orchards (SEDGLEY 1989a; SEDGLEY 1989b; SEDGLEY 1996; SEDGLEY and GRIFFIN 1989).

The flowers of *A. auriculiformis* and *A. mangium* were observed to have nectar on the base of the phyllode; however, the main attractant to visitors may be an abundance of inflorescences at the stage of dehiscence. The visitors are mainly small insects like flies and bees that are able to land on the small flowers grouped into an *Acacia* spike (SEDGLEY *et al.* 1992a). They visit the flowers during the peak of pollen presentation; however, they would promote self-pollination if they have visited different spikes in the same tree before moving to another tree (SEDGLEY *et al.* 1992a). No observations related to the contribution of pollinators and their foraging behaviour to pollination in mixed-ploidy *Acacia* populations have yet been reported.

1.4.4. Breeding system

Acacia mangium and *A. auriculiformis*, like other Australian acacias, have a complex breeding system involving floral morphology, andromonoecy, protogyny and potentially self-incompatibility (SI). A SI mechanism was exhibited in at least 15 other species of acacias (KENRICK 2003).

A report based on isoenzyme analysis confirmed that natural populations of *A. auriculiformis* have a high outbreeding rate (MORAN *et al.* 1989), while studies using DNA microsatellites showed natural populations of *A. mangium* had great variabilty in outcrossing rate, ranging from complete selfing in the extremely low genetic diversity populations (*e.g.* a population in Sidei, Irian Jaya) to complete outcrossing

(*e.g.* Papua New Guinea populations) (BUTCHER *et al.* 2004). Furthermore, HARWOOD *et al.* (2004) established that *A. mangium* was not always self-incompatible in Vietnam; some provenances from two seed orchards had predominantly selfed progeny.

There are no similar studies on *Acacia* polyploids; however, polyploidy is often associated with a break-down of self-incompatibility systems and enhances the selective advantage of floral features that facilitate self-pollination (LEVIN 1983; MABLE 2004; ROSQUIST 2001).

1.5. The problem and potential solution for invasiveness of *Acacia* species.

There are many forest tree species planted in different areas in the world where they are not indigenous; however, they have overcome geographical, environmental, and reproductive barriers and overcome the competition from native species to colonize new locations. The species that are particularly highly adaptable and commercially important pose special challenges to invasiveness. There have been three woody taxa with a long history of widespread introduction and successful planting in many parts of the world outside their native range: *Acacia* Mill., *Eucalyptus* L'He´r. and *Pinus* L. (RICHARDSON *et al.* 2011).

Like eucalypts and pines, human usage and perception has been the driver of *Acacia* species invasiveness, resulting in at least 23 species confirmed as invaders (RICHARDSON and REJMANEK 2011). These include species such as *A. dealbata* Link in South America and Europe (PAULA *et al.* 2010), *A. mearnsii* and *A. saligna* in South Africa (BECK 2004; BLAKESLEY *et al.* 2002; RIITTA *et al.* 2009) and *A. mangium* and *Acacia* hybrid in some countries of southeast Asia (TURNBULL *et al.*

1998). Moreover, features of the reproductive biology of these *Acacia* species, such as pollination systems (*e.g.* massive number of flowers, high attractiveness to flower visitors, early reproductive maturity and longevity), prolific seed production, efficient seed dispersal, and long-lived seed, have also contributed to their invasive success (GIBSON *et al.* 2011). Therefore, WILSON *et al.* (2011) recently suggested strategies for limiting Australian acacia invasion that involve proactive, rather than reactive, management.

Proactive management is to reduce the population size to an acceptable level or to minimize the impact on the ecosystem. It could be either limiting recruitment opportunities (*e.g.* seed dispersal and coppice) by direct management (*e.g.* restriction of seed movement or grazing and fire regimes) or biological control methods, such as harvesting flowers or using biological control agents to reduce flowering and seed set (WILSON *et al.* 2011). However, another alternative, usage of sterile cultivars, was suggested to limit seed production without compromising commercial production (GRIFFIN *et al.* 2011; WILSON *et al.* 2011).

Deployment of triploid (3*x*) clones that were documented to have low fertility or even sterility by crossing tetraploids (4*x*) with diploids (2*x*) was advocated as one of the potential approaches to deal with *Acacia* weediness (BLAKESLEY *et al.* 2002; ZWAAN 1980). Polyploids have occurred in natural population of some other *Acacia* species, such as allotetraploid *A. holosericea* A. Cunn. ex Don and *A. cowleana*; allohexaploid *A. colei* Maslin & Thomson (MASLIN and THOMSON 1992); and a very low frequency of triploid individuals of *A. dealbata* has been found in the Maribyrnong (VIC, Australia) population of this species (BLAKESLEY *et al.* 2002). However, there are no reports of polyploidy in natural populations of *A. mangium* or

A. auriculiformis. The question is whether polyploidy can be used effectively in a forest breeding program.

1.6. The potential for using polyploidy for *Acacia* breeding

The core activities of the breeding cycle of forest tree improvement programs are selection (*e.g.* species, provenance, population, and individual tree) and inter-mating to induce recombination of alleles during sexual reproduction (WHITE T.L. *et al.* 2007).

Polyploidy is a common natural phenomenon in plants and up to 80 % of angiosperm species have undergone genome duplication in their evolutionary history (NUISMER and CUNNINGHAM 2005; RAMSEY and SCHEMSKE 1998). RANNEY (2006) proposed that apart from the profound importance in plant evolution, polyploids are of interest to breeders for utilization in plant improvement. The variations and characteristics of different cytotypes contribute to enlarging flower size, increasing heterosis and hybrid vigor, improving pest resistance and environmental tolerance, overcoming barriers to hybridization, and developing sterile cultivars (LEVIN 1983; RAMSEY and SCHEMSKE 2002; RANNEY 2006). Hence, inducing autopolyploids used for a plant breeding program might be worth considering, particularly in the case of invasive species.

Efforts to induce tetraploidy have been undertaken to support polyploid breeding programs, in *A. mearnsii* (BECK-PAY 2012; MOFFET and NIXON 1975), *A. nilotica* (GARG *et al.* 1996), and *A. dealbata* and *A. mangium* (BLAKESLEY *et al.* 2002). Colchicine-induced autotetraploid individuals of *A. mangium* (AM-4*x*) were subsequently inter-planted with diploids of both *A. mangium* (AM-2*x*) and *A.*

auriculiformis (AA-2*x*) in Vietnam to promote inter-cytotype pollination (KHA *et al.* 2009). However, in a screening of 758 open-pollinated seedlings raised from the seeds that were collected from parent trees at two heavy flowering seasons of all clones in each of the three species/ploidy categories, only three stable 3*x* genotypes, all derived from AA-2*x* mothers, were found (J.L. HARBARD unpublished). Thus, the reproductive behaviour of these two different cytotypes and species needs to be investigated to determine whether there are barriers to the production of triploid progeny within and between these two species.

1.7. Aims of the study

The overall aim of this thesis was to identify whether there are differences in reproductive behaviors of AM-4*x*; AM-2*x* and AA-2*x* contributing to the difficulty of production of 3*x* progenies, either within species or between species. The following specific questions were addressed:

1. Are there any differences in floral phenology and morphology between AM-4*x* and AM-2*x* in comparison with AA-2*x*? If so how do these differences affect the desired inter-cytotype hybridization?
2. How are differences in pollen-pistil interactions involved in determining success of inter-cytotype crosses within and between these two species?
3. Do the factors operating between fertilization and seed maturation limit the production of viable 3*x* progenies from the inter-cytotype crosses?

CHAPTER 2. MATERIALS AND METHODS

This chapter describes materials and methods used for field and laboratory work throughout the study.

2.1. Plant materials

The study was conducted in an *Acacia* orchard established in 2003 at Bau Bang, Binh Duong province in southern Vietnam ($11^{o}15$'N, $106^{o}38$'E, and 50 m elevation). This orchard included 30 putative tetraploid *A. mangium* (AM-4*x*) clones, produced by colchicine induction by Shell Forestry International in England (BLAKESLEY *et al.* 2002) and imported to Vietnam in tissue culture. After weaning, these AM-4*x* clones were established as hedge plants and ramets were propagated by rooting of stem cuttings. Rooted stem cuttings from ten selections of each of diploid *A. mangium* (AM-2*x*) and *A. auriculiformis* (AA-2*x*) from Vietnam's breeding programs were also included in the orchard.

The orchard was established with alternate rows of AM-4*x,* AM-2*x* and AA-2*x* to promote inter-cytotype pollination (Fig. 2.1). Ramets of the different clones were randomized within each row. Clones were set out in single tree plots in the putative AM-4*x* rows and in double-tree plots in the AM-2*x* and AA-2*x* rows. There were four replicates, each with four rows, including two putative AM-4*x* rows and one row each of AM-2*x* and AA-2*x*, with clones in each row separately randomized. The fourth replicate had an additional row of AA-2*x*. Spacing between rows was 4 m and the initial spacing between trees within rows was 2.5 m. The trees had attained an

average height of about 11 m at four years after planting (KHA *et al.* 2009). The orchard was thinned to remove about half of the AM-2*x* and AA-2*x* individuals, reducing competition for the AM-4*x* trees, which were somewhat slower-growing. A total of 188 trees remained at the time observations that commenced at age four years.

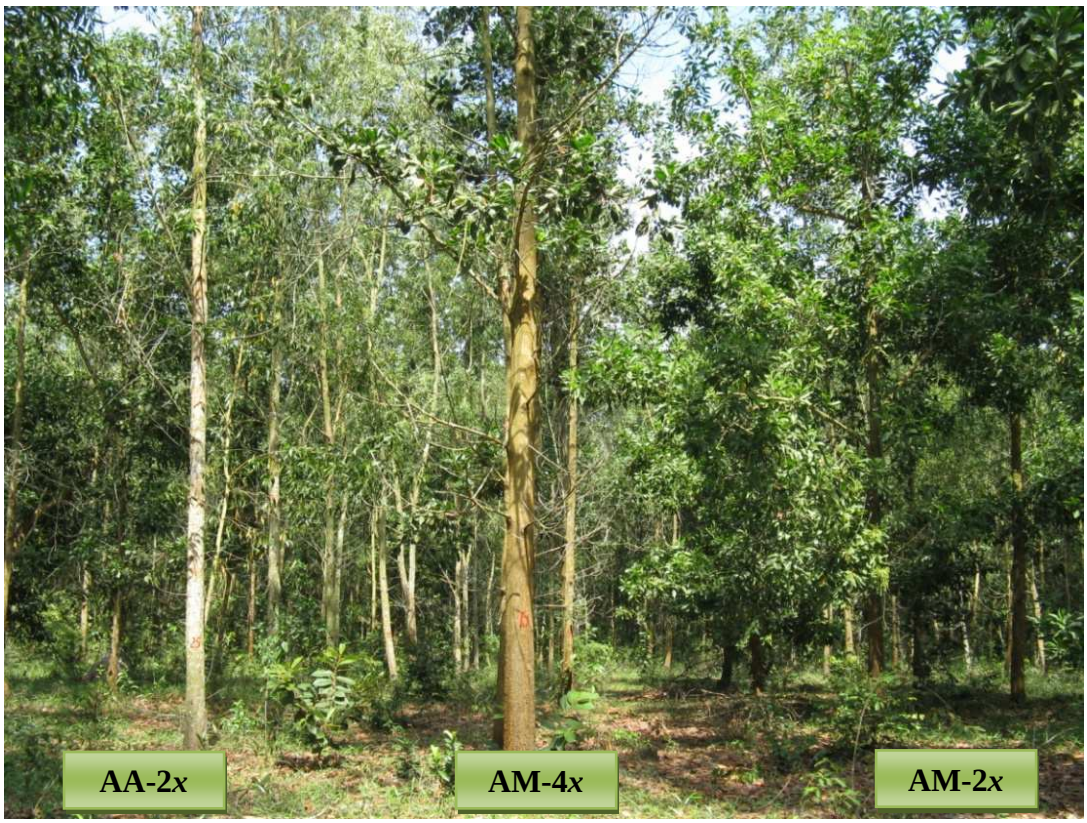


Figure 2.1. The hybridizing orchard in Bau Bang, Southern Vietnam

Prior to this study, the ploidy of each of the putative AM-4*x* ramets was checked by stomatal counts, measurement of polyad diameters and/or flow cytometry (HARBARD *et al.* 2012). This confirmed 38 ramets of 16 clones as tetraploid and the remaining 61 ramets had either reverted to diploid or mixoploid, or remained untested. Data from these 61 trees was omitted from further analysis.

2.2. Hand pollination technique

2.2.1. Pollen collection

Fresh pollen for controlled-pollination was collected from the spikes of selected parent trees which had been bagged in the afternoon of the previous day. The spikes were collected in early morning and dried in desiccators containing silica gel for 3 h, then sieved through a 63 µm stainless steel sieve of (BS4 10/1986, Endecott Ltd. London, England). The fresh pollen sources were divided and stored in cryovial tubes (1.5 ml) with a sufficient amount for one day's pollination.

2.2.2. Hand controlled-pollination

Manual controlled-pollination was conducted based on the standard technique developed by SEDGLEY *et al.* (1992b), including the following steps:

- Spikes were selected in the afternoon of the day before pollination as they changed from green to yellow (Fig. 2.2b)

- A group of spikes were labelled and bagged using three-dimensional polyester pollination bags in three different sizes depending on the size of flower branches (PBS International, United Kingdom).

- The selected spikes were un-bagged the following day and unhealthy and/or unopened spikes were removed. The number of flowers on each spike was reduced to 20 - 30. The remaining flowers were emasculated by removing the stamens using fine forceps (Fig 2.2c).

- A drop of 20 % sucrose solution was applied to each stigma tip before transferring pollen to assist polyad adhesion and germination (GRIFFIN *et al.* 2010).

Pollinated flowers were checked randomly using a handy light scope 30x (Eschenbach, Nürnberg, Germany).

- Spikes were re-bagged for three days and labelled with numbered tags. The bags were temporarily removed for sampling at 24 h and were not replaced after the 72 h sample collection.

Figure 2.2. Hand pollination

(a) scaffolding set up for pollination, (b) spikes at different developmental stages (scale bar = 1 cm), and (c) spike with emasculated flowers ready for applying pollen (scale bar = 2 mm)

2.3. Microscopy

The pollinated flowers were collected at 24 h and 72 h after pollination and fixed in 3:1 methanol: acetic acid solution for a minimum of 3 h. The solution was replaced by 70 % ethanol for transportation.

A clear-squash technique for the study of pollen tube growth was applied (MARTIN 1959), as follows:

- *Hydration*: the ethanol from flower sample preservation was gradually replaced by distilled water through a series of solutions of gradually decreasing ethanol content (70 % and 30 %) until the ethanol was fully replaced by distilled water. Time for each hydration was 20 min, with two final rinses with distilled water.

- *Softening and clearing*: flowers were softened and cleared in 0.8N NaOH for 8 - 9 min in an oven held at 60^{o}C, then rinsed with distilled water after the NaOH had been poured off.

- *Staining*: aniline blue (0.1 %) was used for staining callose for a minimum of 30 min before removing for dissection.

- *Dissection and mounting on a microscope slide*: the flowers were removed from the stain and placed on a petri dish under a binocular dissecting microscope (Zeiss Stemi SV6, Germany). Ten drops of 80 % glycerol were placed on a microscope slide. Five drops were used for the styles and five drops for the corresponding ovaries. Using fine dissection needles the style was separated from the ovary and placed in a drop of glycerol. The ovary was divided in half by slicing along the cleavage and placed in a drop of glycerol. In this manner, five pistils were arranged on each slide. A cover-slip was gently lowered over each group of styles

and ovaries, thereby preventing air-bubbles. The styles were gently squashed by placing pressure on the cover-slip. Slides were sealed by running around the edge of the cover-slip with clear nail varnish.

- *Observation*: the pollen tubes were observed using fluorescence microscopy (Zeiss Axioskop 2, Germany) with UV light at 200x magnification. Filter set 05 with the wavelength of 400 - 445 nm (blue/red light) and condenser lens II were used. The images were digitally captured by Axiovision 3.1 software (Zeiss, Germany).

2.4. Microsatellite markers

Seeds from controlled pollinations were germinated in petri-dishes at 23^{o}C. The germinated seeds were sowed in 6 x 8 pot-trays filled with a potting mix (composted pine bark with washed river sand and slow release Osmocote) in the glasshouse with automatic control of temperature (26^{o}C in day and 20^{o}C night) and watering (twice per day at 11:00 am and 3:30 pm). Propagator lids were used to keep the new sown seed warm for the first week.

DNA of the progeny was extracted from either non-germinated seeds or young phyllodes of three-month old seedlings, depending on the capacity for germination and viability of different types of cross-combinations. DNA from the parents of the studied crosses was extracted from phyllodes that were harvested from the parent trees in Vietnam, and dried in silica gel before transport to Australia.

- *Sample preparation*: The un-germinated seeds were cleaned with distilled water to eliminate fungi before placing each seed into an individual micro-centrifuge tube (1.5 µl) along with a tungsten carbide bead. The tubes were then placed on the Mixer Mill 300 (MM300, Retsch, Germany) and shaken twice for 1 – 2 min at high-

speed (30 Hz) in order to homogenize the seeds. Alternatively, 200 mg of young phyllode tissue from three-month old seedlings or 100 mg of dried phyllode tissue from the parent samples was frozen in liquid nitrogen and homogenized using a mortar and pestle.

- *DNA extraction:* DNA was extracted using the QIAGEN DNeasy^{TM} 96 plant kit (QIAGEN Ltd., United Kingdom). A few adjustments of the DNA extraction procedure were made so that it would work better in *Acacia* (e.g. increasing amount of Buffers AP1 and AP2 in sample extraction routine up to 750 µl and 280 µl, respectively, instead of 400 µl and 130 µl; and adjusting time and temperature for incubation to 25 min at 55^{o}C in place of 10 min at 65^{o}C) (GRIFFIN *et al.* 2012; VUONG 2009).

- *DNA concentration and purity:* the DNA was measured with a NanoDrop 8000 spectrophotometer (Thermo Scientific, Wilmington, DE 19810, USA). One microlitre of each DNA sample could be loaded separately into each well of a standard 96-well micro titer plate to check nucleic acid concentration. The result showed that the nucleic acid concentration of our *Acacia* samples was in a range of 2.5 - 300 ng/µl across the different samples. For polymerase chain reactions (PCRs) the concentration required was a minimum 2.5 ng/µL. Therefore, those samples with high nucleic acid concentrations were diluted with *MilliQ* water to achieve an even concentration range for PCR.

- *Microsatellite method*: Six microsatellite markers developed in earlier studies (BUTCHER *et al.* 2000; NG *et al.* 2005) were used. These were AH16, AH29, Am429, Am436, Am465, and Am030. Primers were synthesized by Sigma Proligo (www.sigma-aldrich.com) and the forward primers of each pair were labeled with

one of three fluorescent dyes. Primer mixes and optimum annealing temperatures of primer mixes were determined using Multiplex Manager 1.0 (www.multiplexmanager.com). PCRs contained 2x QIAGEN Multiplex PCR Master Mix, template DNA, RNase-free water and primers. The PCR mix was mixed thoroughly. Amplification cycles were optimized based on BUTCHER *et al.* (2000) and are presented in Table 2.1

Table 2.1. PCR thermocycle

Step	Temperature (^{o}C)	Time
1	95	15 min
2	94	30 sec (34 cycles)
3	annealing temperature (e.g. 60 and 65^{o}C)	1min 30 sec
4	72	1 min
5	60	15 min
6	72	10 min

PCR products were checked by electrophoretic separation on 1.5 % agarose (TAE) gels and then they were co-loaded into sample loading solution (SLS) mix made up from 200 µl of SLS and 1.25 µl 400 bp size standards per row of 8 wells with an aliquot of 25 µl into each well. Microsatellites were analyzed on a Beckman sequencer (CEQ^{TM}8000) and examined using CEQ Genetic Analysis System software.

By examining the alleles present at each locus, and comparing them with the parental genotypes, it was possible to determine whether each individual progeny was derived from the putative parents, from selfing of the maternal parent, or from a cross involving a non-target male parent.

2.5. Flow cytometry analysis

Although chromosome counting provides the most direct demonstration of ploidy, and was shown to be technically feasible for *A. mangium*, this method was impracticable on the scale required.

Measuring DNA content of nuclei for determining ploidy levels by using flow cytometry (FCM) techniques was selected as the most appropriate method of directly confirming ploidy level. FCM techniques uses the principle of light scattering, light excitation, and emission of flourochrome molecules to generate specific multi-parameter data from particles and cells in the size range of 0.5 to 40 um diameter (http://biology.berkeley.edu) and it could be thus the most accurate method.

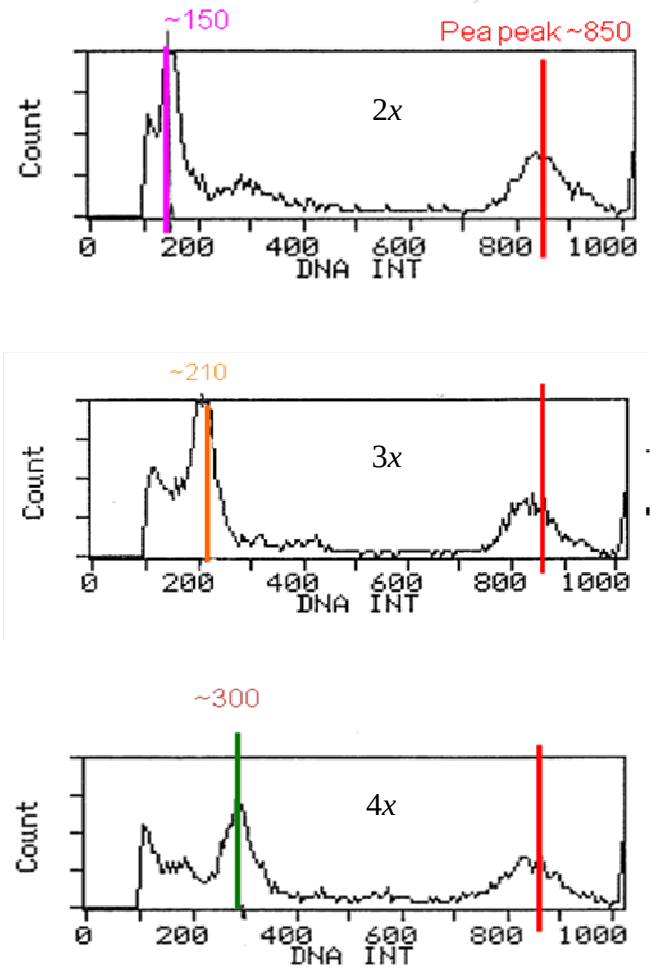


Figure 2.3. Histograms of flow cytometry display the cellular DNA content (light scatter intensity) on the x-axis and the number of cells counted on the y-axis

Nuclei extractions were prepared by chopping 15 mg of fresh phyllode samples or un-germinated seeds on ice in Galbraith buffer (GALBRAITH *et al.* 1983) modified after BEATSON *et al.* (2003). This was combined with a 4.5 % w/v known internal pea standard (*Pisum sativum* cv. Torstag, 2n = 2*x* = 14; 2C DNA amount = 9.10 pg) (BENNETT and LEITCH 1995) prepared in the same buffer. This standard was added to the sample and filtered through a 40 μm nylon cell strainer (BD Falcon). A total of 300 μl of filtrate was mixed with 150 μl of 20 μg/ml propidium iodide stain (Sigma) and samples run on a Coulter Elite ESP Cell Sorter until 10,000 events were collected using a 488 nm air cooled argon laser (Uniphase, San Jose, CA, USA) and 575 nm band pass filter.

DNA ploidy status of samples was defined based on the amount of DNA relative to the pea control. Single distinct peaks were seen at channels (*i.e.* 850 for pea and ~ 150 for 2*x*; ~ 210 for 3*x*; and ~ 300 for 4*x*) (Fig. 2.3).

CHAPTER 3. FLORAL PHENOLOGY AND MORPHOLOGY

This chapter explores whether differences in floral phenology and morphology between tetraploid *A. mangium* (AM-4*x*) and diploids of both *A. magium* (AM-2*x*) and *A. auriculiformis* (AA-2*x*) are likely to contribute to the low yield of triploid progenies and the high selfing rates of AM-4*x* (GRIFFIN *et al.* 2012). These were observed in the Bau Bang orchard where the rows of AM-4*x*, AM-2*x* and AA-2*x* were inter-planted to promote the production of open pollinated triploid seeds from inter- and intra-specific mating.

3.1. Introduction

Acacia mangium and *A. auriculiformis* have played an important role in production forestry, and understanding the reproductive biology of these species is extremely important to achieve success of an inter-mating scheme for a breeding cycle of a tree improvement program. There have been a numerous studies on floral phenology and morphology, pollination mechanism, and breeding system of *Acacia* species in general as well as of *A. mangium, A. auriculiformis* and their hybrid in particular as summarized in Chapter 1. Based on these investigations, the technique of hand cross-pollination for acacias was developed (GRIFFIN *et al.* 2010; SEDGLEY *et al.* 1992b).

No studies have yet described how reproductive biology is affected by different cytotypes within these species. Since reduction of fertility of acacias is of interest to breeders the deployment of sterile triploid (3*x*) clones is one possible approach (BECK-PAY 2012; BLAKESLEY *et al.* 2002; GRIFFIN *et al.* 2011; ZWAAN 1980).

Polyploidy, genome duplication, is a common natural phenomenon in the evolutionary history of plants (NUISMER and CUNNINGHAM 2005; RAMSEY and SCHEMSKE 1998). There is thus no exception in acacias as some taxa of the genus *Acacia* are known to be polyploid, especially since very low frequency of tetraploid and triploid individuals of *A. dealbata* has been found (BLAKESLEY *et al.* 2002). These observations have provided some confidence in induction of polyploids of *A. mearnsii* (MOFFET and NIXON 1975), *A. dealbata* and *A. mangium* (BLAKESLEY *et al.* 2002). The induced autotetraploid clones of *A. mangium* were used as the initial material from which a Vietnamese polyploid breeding program has been developed (KHA *et al.* 2009).

Commencing in 2007, many kilograms of seed from each of the three species/ploidy combinations in Vietnam hybridising orchard were harvested, representing hundreds of thousands of viable seeds. A separate study undertaken to examine breeding systems in this orchard reported that eight clones of diploid *A. mangium* were predominantly (~ 97 %) outcrossing whereas six clones of tetraploid *A. mangium* had an extremely high selfing rate (average of 98 %) (GRIFFIN *et al.* 2012; VUONG 2009). Increased levels of selfing in the tetraploid may be driven by genome doubling which typically results in a breakdown of self-incompatibility (MABLE 2004) and significant differences in cell size, cell activity, physiology, development and reproductive system when compared with their diploid relatives (LEVIN 1983). In this chapter, we examine whether such differences in floral phenology and morphology between diploid and tetraploid *A. mangium* are significant barriers to the desired inter-cytotype hybridizations and production of triploid individuals as well as involved in the high selfing rates of AM-4*x* clones.

3.2. Materials and methods

3.2.1. Phenology study

A total of 127 trees in the orchard, including 38 confirmed AM-4*x*, 23 AM-2*x* and 66 AA-2*x* ramets, were observed at fortnightly intervals from October 2008 to April 2010. At each observation, flowering and fruiting intensity was scored visually using the following categories for intensity:

0 - no flowers, fruits

1 - up to ⅓ of crown bearing open flowers, mature fruits

2 - ⅓ to ⅔ of crown bearing open flowers, mature fruits

3 - more than ⅔ of crown bearing open flowers, mature fruits.

To obtain the monthly flowering and fruiting intensity as defined by ZAKARIA and KAMIS (1992), the scores of all trees per species/ploidy combination, for each month, were summed and divided by the total number of trees x 3 (highest intensity) and expressed as percentage of full flowering or fruiting intensity.

Climatic data recorded by the Binh Duong Meteorological Bureau was obtained from So Sao station (15 km from the study site). Mean monthly maximum and minimum temperature (^{o}C) varied little over the year. The dry season extended from November to April, with a distinct rainy season from May to October (Fig. 3.1).

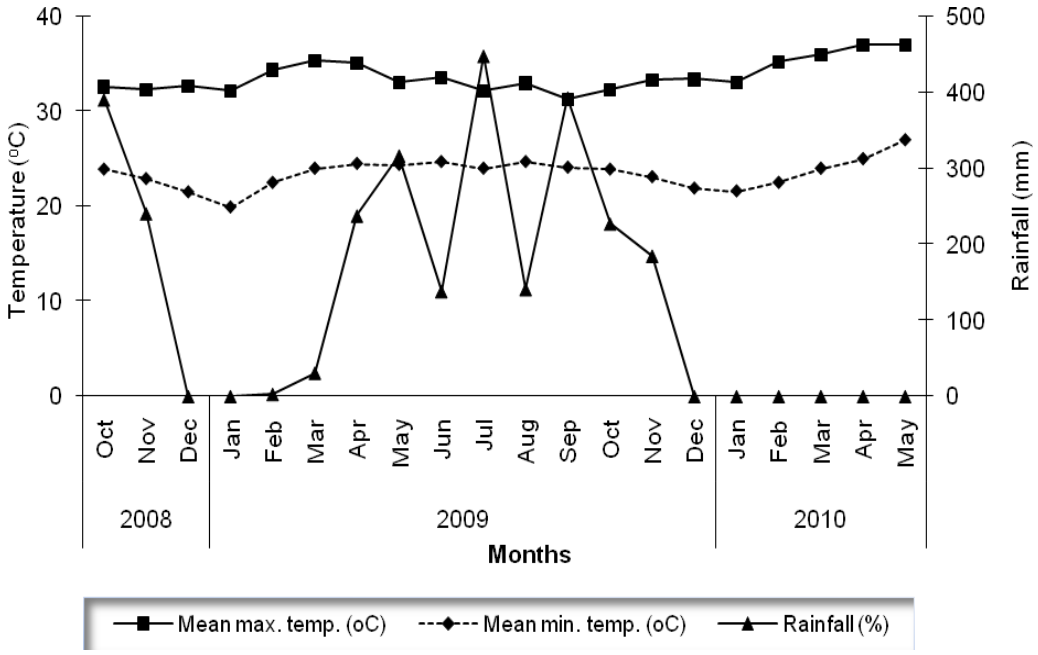


Figure 3.1. Climatic parameters recorded over two studied years

3.2.2. Floral morphology

3.2.2.1. Spike and flower parameters

Flowers of *A. mangium* and *A. auriculiformis* are clustered on cylindrical spikes (inflorescences) that are paired in the upper axils (Fig. 3.8). Three spikes per ramet, from one ramet of each of 10 different clones of each species/ploidy combination (AM-2*x*, AM-4*x*, and AA-2*x*) were collected at anthesis in each of two consecutive years (2008 and 2009). However, one clone of each of the AM-2*x* and AM-4*x* sets was excluded following the first flowering/ fruiting season and replaced by a different clone for the second season. Flower samples were fixed in 3: 1 methanol: acetic acid solution for a minimum of 3 h. The solution was replaced by 70 % ethanol for transportation.

Length of each spike was measured using a ruler graduated in mm, and each flower of every spike was observed using a binocular dissecting microscope (Zeiss Stemi SV6) to score for the presence or absence of a full developed pistil.

Each year, 30 flowers per clone from 10 different clones of each species/ploidy combination were measured for the length of flower and style using the dissecting microscope at 20x magnification. Another 30 flowers per clone were softened, cleared and stained in aniline blue before removal for dissection as described in Chapter 2. Each style and ovary was viewed by fluorescence microscopy (Zeiss Axioskop 2) under UV light at 200x magnification to measure the diameter of the stigma and to count the number of ovules. The diameters of 30 polyads per ramet per clone were also measured using light microscopy (Zeiss Axioskop 2) at 200x magnification. All necessary images were digitally captured using Axiovision 3.1 software (Zeiss, Germany).

3.2.2.2. Pollen collection and germination

Pollen from at least 20 spikes was collected from one ramet per clone of five different clones of each species/ploidy combination, and dried in desiccators containing silica gel for 3 h, then sieved through a stainless steel sieve of 63 µm aperture mesh (BS4 10/1986, Endecott Ltd. London, England). Pollen was placed onto a medium of 1 % agar, 20 % sucrose and 0.01 % boric acid at ambient temperature (about 26^{o}C) for determination of polyad germination and number of pollen tubes per polyad. Germination percentage was recorded by examining three replicates of about 300 polyads per clone by light microscopy (Zeiss Axiolab) after 4h. A polyad was scored as having germinated when the length of at least one pollen tube was longer than the polyad diameter.

3.2.2.3. Pod harvest and seed classification

Thirty individual pods from one ramet per clone of 10 different clones of each species/ploidy combination were harvested when the pods were brown and beginning to dehisce. Each pod was put into a separate plastic bag and the seeds were extracted inside the bag. All seeds within the pod were examined and classed as either unfilled (*e.g.* abnormal size and empty or wrinkled appearance) or filled (*e.g.* normal size and full appearance). The number of filled and unfilled seeds per pod was recorded. Length and width of filled seeds from each of 30 pods from one ramet of five different clones per species/ploidy combination were measured using an electronic calliper (IP54 series – Sheffield, England). The seed samples were weighed to calculate mean seed weight for each combination.

3.2.3. Insect visitors

Observations of floral visitors in the orchard were undertaken during a flowering peak of both *A. mangium* and *A. auriculiformis* in November 2009. Flowering branches of 1 - 3 trees of each species/ploidy combination were observed at close range, from scaffolds adjacent to the trees. Species of insects, timing of insect visits and climatic conditions were recorded.

At least three insects were caught from trees of AM-2*x*, AM-4*x* and AA-2*x* in the morning between 07h30 and 09h30, when the insects tended to forage most intensely. Collected insects were stored (1 - 2 insects per bottle) in bottles filled with 70 % ethanol to enable polyad preservation for later study. Insects were allocated to 1 - 3 bottles per tree, depending on when they were caught. In the laboratory, polyads

were dislodged from the insect bodies into the ethanol solution by shaking the bottle gently.

The diameters of 50 polyads per bottle were determined as previously described. Mean polyad diameters observed in the floral morphology study were used as a guide to assign individual polyads to species/ploidy combinations as follows:

AM-4*x*: polyad diameter 40.5 - 44.6 µm

AM-2*x*: polyad diameter 32.1 - 34.4 µm

AA-2*x*: polyad diameter 35.8 - 38.2 µm

3.2.4. Statistical analysis

Univariate analyses of the studied spike, flower and seed parameters were carried out using the following fixed-effects model:

$$Y_{ij} = \mu + TAXON_i + YEAR_j + (TAXON_i * YEAR_j) + \varepsilon_{ij} \qquad (1)$$

where µ= overall mean, TAXON is the fixed effect of species/ploidy combination (three levels, AM-4*x*, AM-2*x* and AA-2*x*), YEAR is the fixed effect of year of study (two levels, 2008 and 2009), TAXON*YEAR is the interaction between TAXON and YEAR and ε_{ij} is the residual error.

For stigma, polyad and seed size, which was studied in only the first year, model (1) simplified to:

$$Y_i = \mu + TAXON_i + \varepsilon_i \qquad (2)$$

where µ= overall mean, TAXON is the fixed effect of species/ploidy combination and ε_i is the residual error.

Differences in pollen germination between species/ploidy combinations were tested using the following model:

$$Y_i = \mu + TAXON_i + (TAXON_i*CLONE_j) + \varepsilon_{ij} \quad (3)$$

where TAXON*CLONE is the fixed effect of clone within species/ploidy combination.

Square-root transformations of the original data were applied for percentage of male flowers, where plots of residuals versus predicted values showed a non-normal distribution. The significance of differences between different pairs of the TAXON treatments was tested using the Tukey-Kramer method for multiple comparisons, for $\alpha = 0.05$. Analyses were conducted using SAS (version 9.2, SAS Institute, Cary, NC, USA).

3. 3. Results

3.3.1. Phenology

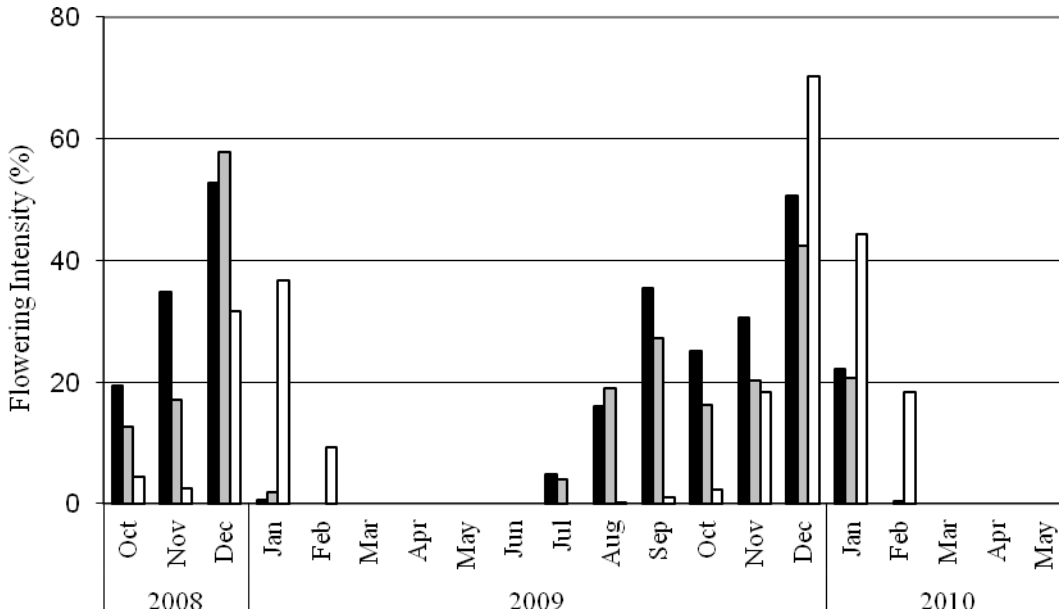
Records of flowering for the 2-year study were made for 127 trees (38, 23 and 66 trees of AM-4*x*, AM-2*x* and AA-2*x*, respectively). Neither *A. mangium* nor *A. auriculiformis* flowered continuously over the study period. In general, the peak flowering periods of the two species started at the end of the rainy season (November - December), though *A. auriculiformis* normally flowered slightly later than *A. mangium*. Flowering lasted for several months with a one-month peak, in November to December for *A. mangium* and in December - January for *A. auriculiformis* (Fig. 3.2 a). Flowering intensity of AM-4*x* and AM-2*x* was similar through the years whereas that of AA-2*x* increased considerably in 2009 (70 % compared with 37 % in 2008). In the 2009 flowering season, AM-2*x* and AM-4*x* had two flowering peaks,

one in the middle of September and the other from November to December, while AA-2*x* had only one peak in December to January. Flowering intensity of *A. mangium* at the first flowering peak was slightly lower than at the second peak, 27 and 35 % in comparison with 43 and 51 % for AM-4*x* and AM-2*x*, respectively.

Pod development occurred during the dry season and the period with the greatest presence of both immature and ripe pods was from March to early May. It appeared that the September flowering resulted in little fruit set in February. At the time when pods were harvested for study, the fruiting intensity of AM-4*x* was similar in both years (~ 36 %), while AM-2*x* and AA-2*x* displayed higher peak fruiting intensities in 2009 (54 – 68 % compared with 36 – 51 % in 2008) (Fig. 3.2 b).

Examination of the fortnightly flowering phenology of individual clones (data not present) showed that during all fortnightly periods when AM-4*x* clones were flowering, there were some AM-2*x* clones also flowering, but *A. auriculiformis* flowered slightly later than *A. mangium*. There was thus a period of 2 - 4 weeks in January - February in both 2009 and 2010 where *A. auriculiformis* was still flowering, but all *A. mangium* had finished flowering.

(a)



(b)

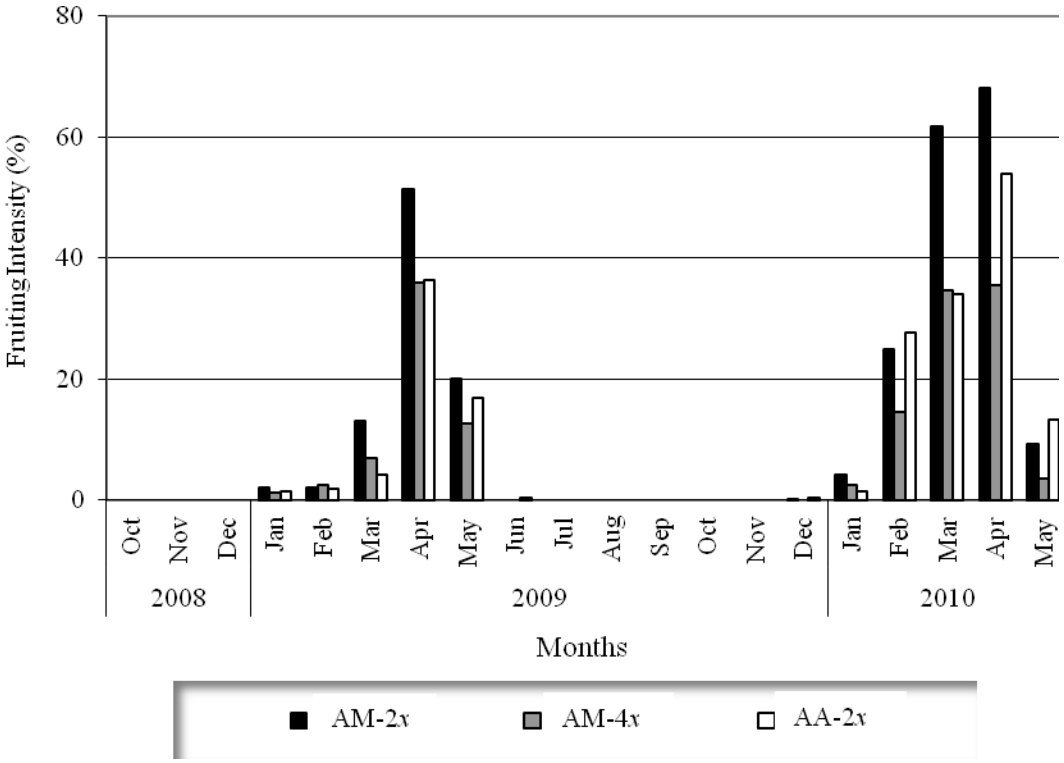


Figure 3.2. Monthly mean flowering/ fruiting intensity (%)

3.3.2. Floral morphology

AM-4*x* spikes were similar in general appearance to AM-2*x* spikes, both being cream colored while flower spikes of *A. auriculiformis* were yellow (Fig. 3.8). Individual flowers were arranged along a spike with both hermaphrodite and staminate flowers. The hermaphrodite flower has a single pistil and a large number of stamens (Fig. 3.3a). There were significant differences in spike length, the total number of flowers per spike and the percentage of male flowers per spike ($P \leq 0.001$) among species/ploidy combinations. However, no significant differences were observed between years (2008 and 2009), except for the percentage of male flowers per spike (Table 3.1).

AM-4*x* had shorter flower spikes (8.0 - 8.7 cm compared with 9.9 - 10.8 cm) than AM-2*x*, but its spikes were longer than AA-2*x* (~ 5.8 cm). The mean number of flowers per spike was less for AM-4*x* (161.4 - 199.3 flowers) than for AM-2*x* (204 - 230.4 flowers). *Acacia auriculiformis* (90.4 - 99.4 flowers) had only about half the number per spike, compared with *A. mangium* (Table 3.1). Taxon-by-year interactions were statistically significant for all spike parameters ($P \leq 0.01$ or 0.001). There were rank changes from year to year for the percentage of male flowers, but not for spike length or total number of flowers per spike.

There was a similar percentage of male to hermaphrodite flowers, with less than 23 % male flowers for all three species/ploidy combination in both study years. However, this percentage was variable from year to year, with AM-2*x* greater than AM-4*x* in 2008 (20 % compared with 11 %) but less in 2009 (5 % compared with 17 %) (Table 3.1). The high percentage for AM-2*x* could be due to one of the 10 studied clones having mostly male flowers (67.8 %) in 2008 and just 3.8 % in 2009 (data not

presented). In a follow-up study in the orchard in 2010, male flowers were observed to be distributed randomly along the spike in each species/ploidy combination (M. HANSON and J.L. HARBARD, pers. comm.).

Table 3.1. Mean values of spike length, total number of flowers per spike, and percentage of male flowers per spike for three species/ploidy combinations over two studied years

Species/ploidy combination	Spike length (cm)		Total number of flowers per spike		% of male flowers per spike	
	2008	2009	2008	2009	2008	2009
A. mangium 2*x*	9.9 [a]	10.8 [a]	204.1 [a]	230.4 [a]	20.3 [a]	4.9 [b]
A. mangium 4*x*	8.7 [b]	8.0 [b]	199.3 [a]	161.4 [b]	11.2 [b]	16.7 [a]
A. auriculiformis 2*x*	5.7 [c]	5.8 [c]	90.4 [b]	99.4 [c]	22.5 [a]	21.2 [a]
Differences between years	n.s.		n.s.		***	
Taxon x Year interaction	**		***		**	

Note: n.s. = not significant, ** = $0.001 \leq P \leq 0.01$ and *** = $P \leq 0.001$.

Letter for significance of differences of the species/ploidy combination within years (using Tukey-Kramer significant differences at $P < 0.05$).

Species/ploidy combinations differed significantly ($P \leq 0.001$) in their flower and style length and these differences were consistent across years. The flower and style length of AA-2*x* was considerably shorter than that of both AM-4*x* and AM-2*x*. The mean style and flower length for AA-2*x* was3.4 - 4.3 mm and for AM-2*x* 4.2 - 5.1 mm and AM-4*x* 3.9 - 4.8 mm in the 2 studied years (Table 3.2). However, AA-2*x* had more (16) ovules per ovary, compared with AM-4*x* and AM-2*x* (13 and 14, respectively) (Table 3.2). Neither style length nor ovule number differed significantly between years.

Table 3.2. Mean values for flower length, style length and number of ovules per flower for three species/ploidy categories over two studied years

Species/ploidy	Flower length (mm)	Style length (mm)	Number of ovules per ovary
A. mangium 2*x*	5.1 [a]	4.2 [a]	14.2 [ab]
A. mangium 4*x*	4.8 [a]	3.9 [b]	13.0 [b]
A. auriculiformis 2*x*	4.3 [b]	3.4 [c]	15.5 [a]
Difference between years	n.s.	n.s.	n.s.
Taxon x Year Interaction	n.s.	n.s.	n.s.

Note: n.s. = not significant. Letter for significance of differences of the species/ploidy combination within years (using Tukey-Kramer significant differences at $P < 0.05$).

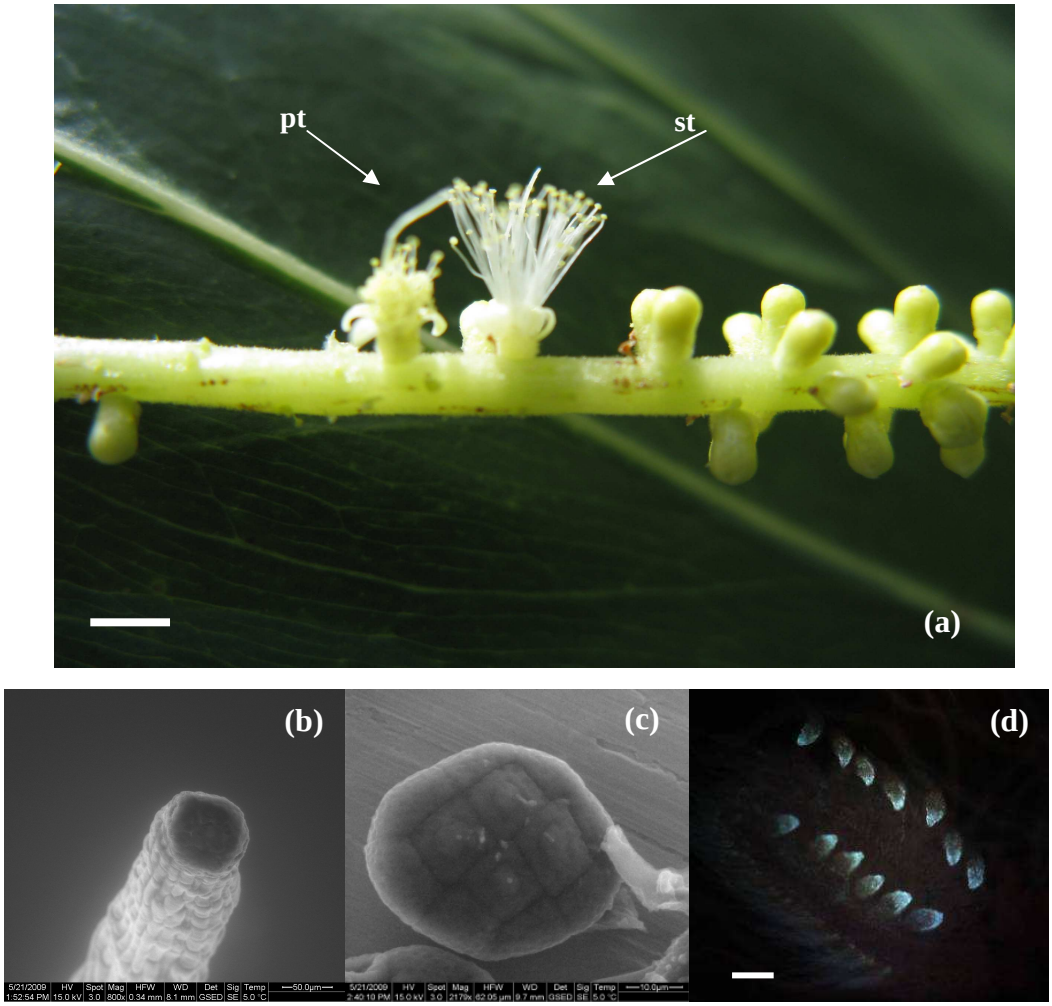


Figure 3.3. Flower morphology

(a) a flower with one pistil (pt) and a number of stamens (st) (scale bar = 2.5 mm); (b) the stigma (scale bar = 50 µm); (c) polyad (scale bar = 10 µm); and (d) the ovules (scale bar = 50 µm).

AM-4*x* had significantly greater mean stigma diameter, 62 µm, compared with 48 - 51 µm for AM-2*x* and AA-2*x* and significantly greater mean polyad diameter, 42 µm, compared with 33 µm for AM-2*x* and 37 µm for AA-2*x*. However, the width of the stigma cup was always greater than that of the polyad for all species/ploidy combinations, enabling at least one polyad to be accommodated on the stigma regardless of the combination involved (Fig. 3.4 & 3.5).

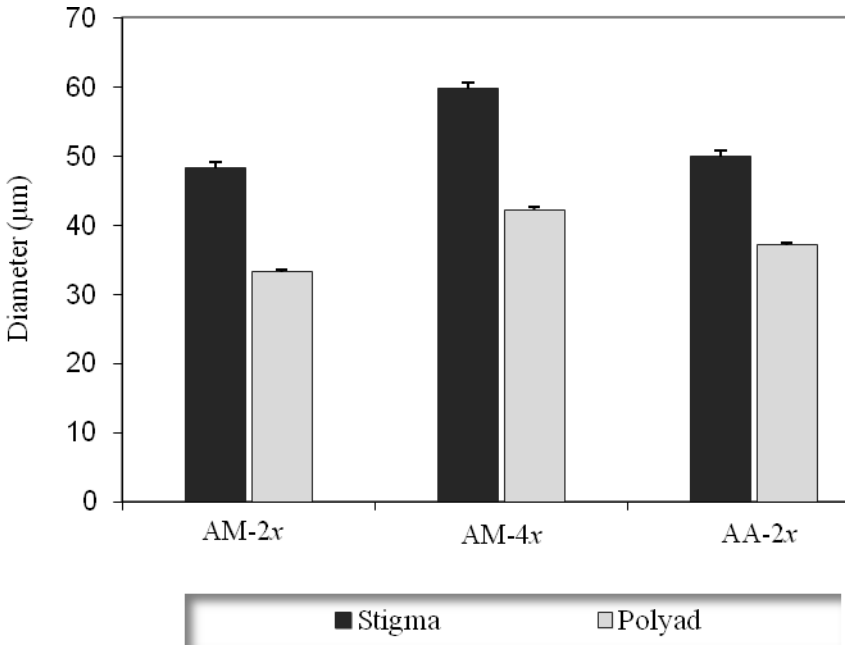


Figure 3.4. Mean diameter of stigmas and polyads for AM-2*x*, AM-4*x* and AA-2*x*. (Error bars demonstrated critical differences ($P < 0.05$) between taxa)

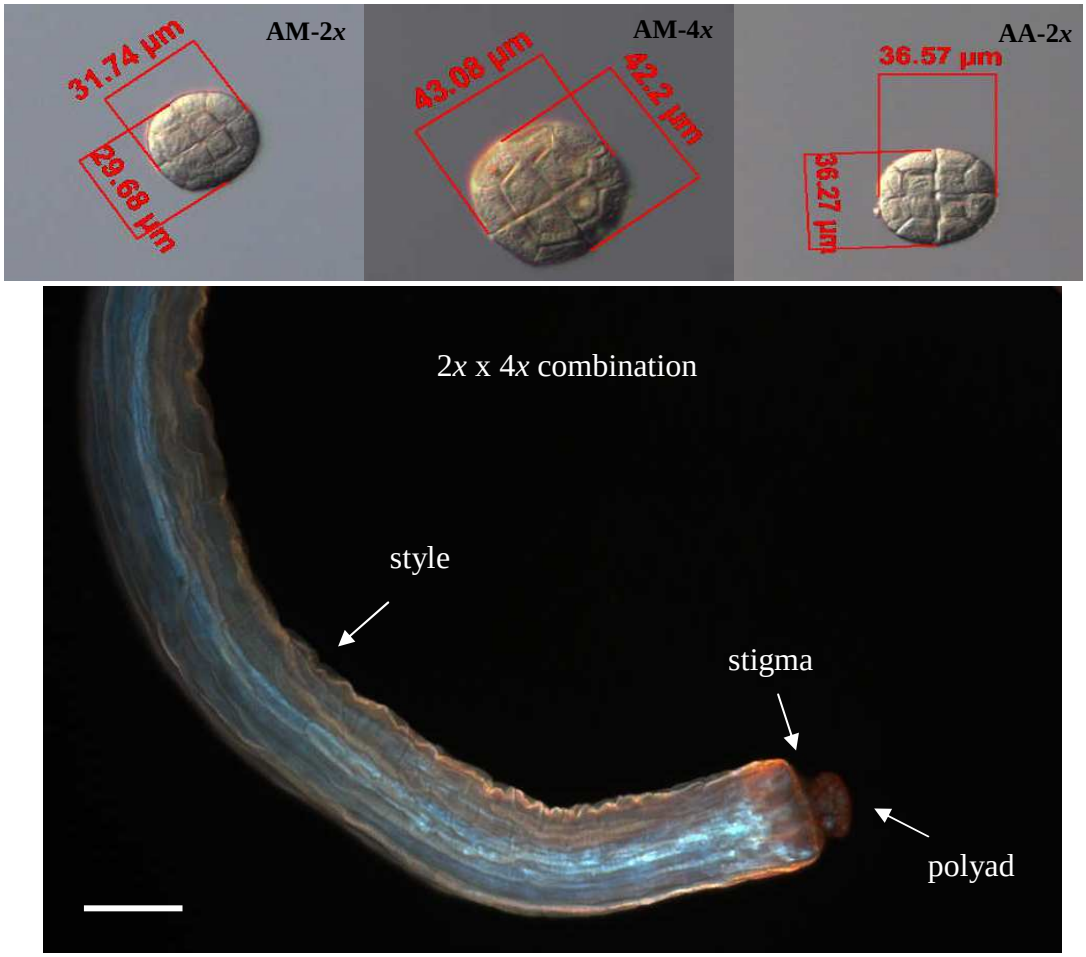


Figure 3.5. Polyads and stigma-polyad combination (scale bar = 50 µm)

3.3.3. Pollen quality

There was no significant difference in mean percentage of pollen germination between 2*x* and 4*x* polyads, with ~ 51 % of AM-2*x* and AA-2*x* and 38.6 % of AM-4*x* polyads germinating (Table 3.3). The lack of a significant difference between 4*x* and 2*x* arose because of major differences in germination percentage among clones within species/ploidy combinations which ranged from 33.1 to 45.2 % for AM-4*x* clones, 22.7 to 82.4 % for AM-2*x* clones and 28.2 to 95.4 % for AA-2*x* clones (data not presented).

Table 3.3. *In vitro* pollen germination of fresh pollen and percentages of germinating polyads with different numbers of tubes per polyad

Species/ploidy combination	Germinating polyads (%)	% of germinating polyads with different numbers of pollen tubes							
		1	2	3	4	5	6	7	≥ 8
A. mangium 2*x*	49.4	22.3	18.5	20.2	17.3	11.4	6.6	2.6	1.2
A. mangium 4*x*	38.6	29.5	23.6	23.8	16.0	6.8	0.3		
A. auriculiformis 2*x*	50.6	7.9	16.9	26.4	16.9	10.2	9.2	5.8	6.7
Difference among taxa	n.s.								
Differences between clones within taxa	***								

Note: n.s. = not significant, and *** = P ⩽ 0.001

However, the number of pollen tubes emerging from each polyad varied among AM-4*x*, AM-2*x* and AA-2*x*. Of the polyads examined, 6.7 % of AA-2*x* produced more than eight pollen tubes, while that percentage of AM-2*x* and AM-4*x* had maxima of six and five pollen tubes per polyad, respectively (Table 3.3 and Fig. 3.6). The most commonly observed numbers of pollen tubes per polyad ranged from 1 to 4 for all three species/ploidy combinations.

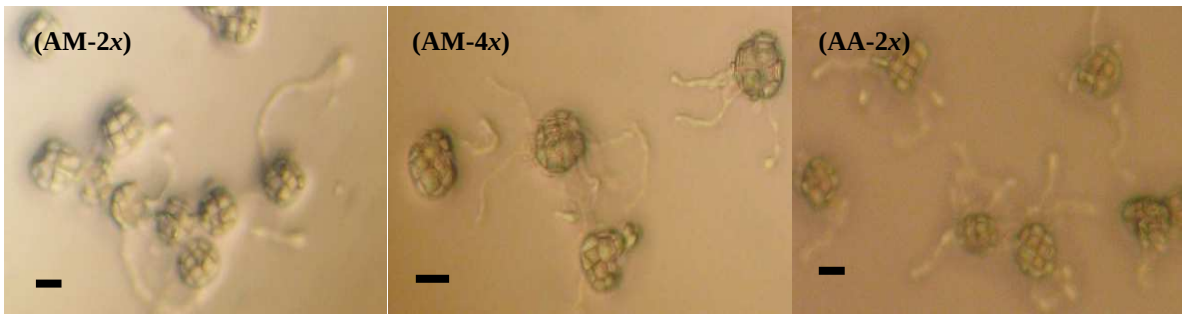


Figure 3.6. *In vitro* polyad germination (scale bars = 25 μm)

3.3.4. Insect visitors

The inflorescences of *A. mangium* and *A. auriculiformis* in the hybridising orchard were visited by honeybees (*Apis mellifera*), native bees (*A. florae*) and bee flies (*Bombyllius major*) (Fig. 3.9). Honeybees were the most common visitors in the orchard. The bees foraged from early to mid morning when anthers dehisce. Honeybees had large pollen baskets on their hind legs visible to the naked eye, whereas no pollen was observed on the beeflies that were collected (Table 3.4).

Polyads of all three types were found on bees visiting one AM-2*x* and one AA-2*x* tree, while only AM-4*x* and AA-2*x* polyads were found on bees visiting an AM-4*x* tree and one AA-2*x* tree. Bee visitors to the third AA-2*x* tree in early morning carried only AA-2*x* pollen (Table 3.4).

Table 3.4. Percentage of polyad types on bees sampled from individual trees

Species/ ploidy	Tree ID	Types of insect	Time of day (h)	Percentage of polyad types		
				AM-2*x*	AM-4*x*	AA-2*x*
A. mangium 2*x*	68	honeybees	08h45	66	4	30
		honeybees	09h15	62	2	36
A. mangium 4*x*	51	honeybees	09h00	0	72	28
A. auriculiformis 2*x*	6	honeybees	09h10	0	46	54
	18	honeybees	07h50	8	44	48
	156	honeybees	07h30	0	0	100
		bee flies	07h50	0	0	0

3.3.5. Yield of mature seed

In two successive years, both AM-2*x* and AA-2*x* set significantly more total seeds per pod (8 - 10 and 7 - 8 seeds per pod, respectively), than did AM-4*x* (~ 4 seeds per pod) (Fig. 3.7).

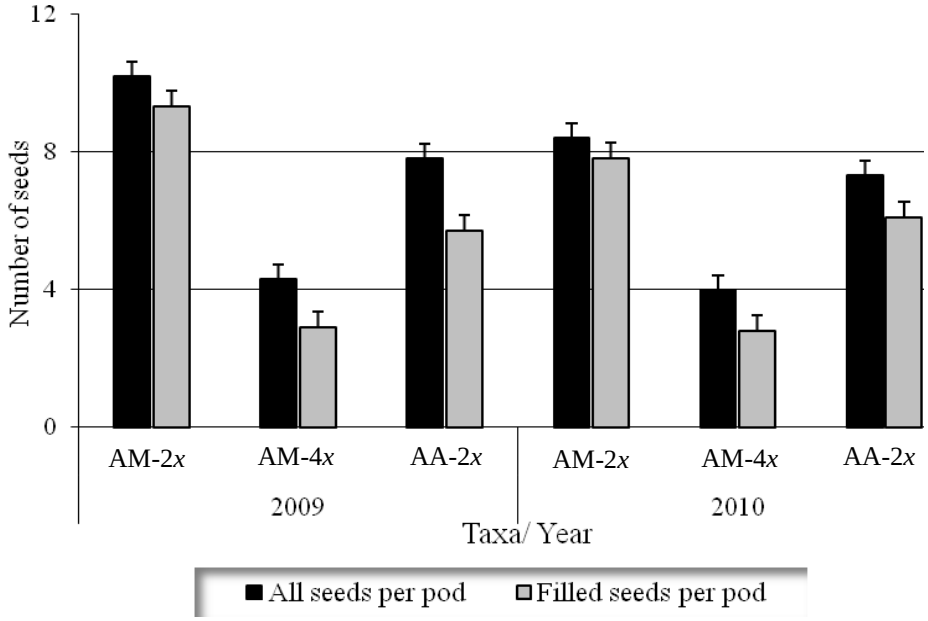


Figure 3.7. Mean total number of seeds and filled seeds per pod
(Error bars demonstrated significant differences ($P < 0.05$) between taxa)

Filled seeds per pod showed the same trend. AM-4*x* that yielded fewer than three filled seeds per pod was less than half that of AM-2*x* and AA-2*x* (7 - 8 and 5 - 6 filled seeds per pod, respectively) (Fig. 3.7). There were no significant differences between years for either trait.

AM-4*x* did not differ significantly in seed length and width from AM-2*x*, but had significantly ($P < 0.05$) greater seed weight (Table 3.5). AM-2*x* and AM-4*x* both had seeds that were significantly ($P < 0.05$) narrower and lighter than those of *A. auriculiformis*, although seed lengths did not differ significantly. Average seed weight of AM-4*x* was 1.45 mg; intermediate between AM-2*x* (1.23 mg) and AA-2*x* (1.69 mg) (Table 3.5).

Table 3.5. Mean values for seed size of three species/ploidy combinations

Species/ploidy	Width (mm)	Length (mm)	Weight (mg)
A. mangium 2*x*	2.8 [b]	4.2 [a]	1.23 [c]
A. mangium 4*x*	3.0 [b]	4.3 [a]	1.45 [b]
A. auriculiformis 2*x*	3.5 [a]	4.6 [a]	1.69 [a]
Difference among taxa	n.s.	n.s.	*
Differences between clones within taxa	n.s.	n.s.	n.s.

Note: n.s. = not significant, * = $P \leq 0.05$.

Letter for significance of differences between species/ploidy combinations within years (using Tukey-Kramer significant differences at $P < 0.05$).

Figure 3.8. Spikes (scale bars = 4 cm)

Figure 3.9. Bees searching on the spikes

3.4. Discussion

Five types of mating combination are possible in the Bau Bang orchard: (i) selfing of each of AM-4*x*, AM-2*x* and AA-2*x*; (ii) intra-cytotype outcrosses within each of AM-4*x*, AM-2*x* and AA-2*x*; (iii) intra-cytotype outcrosses between AM-2*x* and AA-2*x*; (iv) inter-cytotype outcrosses between AM-4*x* and AM-2*x*; and (v) inter-cytotype outcrosses between AM-4*x* and AA-2*x*. The proportions of these crosses occurring in the orchard can be expected to affect yields of mature pods and numbers of seeds set per pod.

Although heavy seed crops collected from all three species/ploidy combinations from 2007 onwards displayed high viability, the yield of viable open-pollinated triploid (3*x*) seeds from open pollinated seed was very low (J.L. Harbard unpublished data). Therefore, barriers to inter-cytotype crossing could be operating within the reproductive cycle.

3.4.1. Phenology

Phenological investigation indicated no effects of cytotypes on the phenophase of *A. mangium*. The flowering intensities of the populations of AM-4*x* and AM-2*x* were very similar, and clones of both cytotypes peaked in December and completed flowering in January (Fig. 3.2a). The secondary flowering peak was observed for both AM-2*x* and AM-4*x* in September 2009 but cannot be confirmed as an annual occurrence, because the first-year observations commenced in October 2008. The fruiting intensity patterns suggest the absence of such a peak in September 2008, as there were very low levels of mature fruit in February 2009, compared with a higher

level of fruit set February 2010, which may have been set during the September 2009 flowering peak (Fig. 3.2b).

Flowering periods for *A. mangium* and *A. auriculiformis* in Southern Vietnam differed regardless of cytotypes in *A. mangium*, since flowering peaks were September - December for *A. mangium* and November - February for *A. auriculiformis*. This is consistent with several studies of the flowering times of diploid *A. mangium* and *A. auriculiformis* in several South-east Asian countries. In these studies, *A. auriculiformis* has typically flowered somewhat later than *A. mangium*. In Malaysia, flowering peak is January for *A. mangium* and July to August for *A. auriculiformis*, while in Taiwan, flowering peak is October - November for *A. mangium* and July - November for *A. auriculiformis* (ZAKARIA and KAMIS 1992). Differences in peak flowering time among *A. auriculiformis* provenances in Thailand have been reported, with provenances from Queensland, Australia and Papua New Guinea having later flowering peaks than those from the Northern Territory (JIWARAWAT *et al.* 1996).

However, in Vietnam, as in other countries, there is a substantial overlap in flowering times between the two species (Fig. 3.2a). Spontaneous inter-specific hybrids observed in the seed from AM-2*x* and AA-2*x* collected from Bau Bang indicate that flowering phenology is not a strong barrier to inter-specific hybridization. As AM-2*x* and AM-4*x* have virtually identical flowering phenology at Bau Bang, it can safely be assumed that flowering phenology does not pose a barrier to production of triploids, either within species (AM-3*x*) or inter-specific triploid hybrids.

3.4.2. Floral morphology

The floral morphology of AM-4*x* was similar to that of AM-2*x* and AA-2*x*, previously described by SEDGLEY *et al*. (1992*c*). Individual flowers are arranged along the flower spike with both hermaphrodite and staminate flowers. Flowers are peduncles, the calyx is 0.6 - 0.8 mm long with short obtuse lobes and the corolla is twice as long as the calyx (ORCHARD and WILSON 2001). The key differences between 4*x* and 2*x* were size related; doubling of chromosome number in tetraploid plants has been observed to produce changes in cell size compared with the original diploid in many species (LEVIN 1983).

AM-4*x* differed from AM-2*x* in having significantly shorter spike length, flower and style length, and smaller flower numbers per spike (Tables 3.1 and 3.2). The significant decline in the number of AM-4*x* flowers per spike appears to be a consequence of the shorter spike length ofAM-4*x* compared withAM-2*x*. The AA-2*x* spike was the shortest and had the smallest number of flowers per spike, consistent with previous research on the floral morphology of diploid *A. mangium* and *A. auriculiformis* in Malaysia and Australia (SEDGLEY *et al.* 1992d; ZAKARIA and KAMIS 1991). These studies reported that *A. mangium* had significantly longer spike, flower and style length as well as more flowers per spike than *A. auriculiformis*. However, this morphological difference was not a strong barrier to 2*x* inter-specific crossing since their spontaneous hybrids are widespread when exotic plantations of the two species are grown together. Because AM-4*x* morphological traits were shown to be intermediate between the two diploid species (Tables 3.1 and 3.2), it is concluded that morphological differences are unlikely to affect crossing with either diploid.

An effect of floral morphology on open-pollination efficiency was reported for different populations of *A. senegal* in which the open-pollination efficiency was higher (36 – 40 %, compared with 24 %) in the longer-styled (10.2 mm) than in the shorter-styled (7.3 mm) populations (TANDON *et al.* 2001). They suggested that long styles may be more suited to some insect than short styles for 'combing' pollen from the insect body. However, the slightly shorter style length of AM-4*x*, relative to AM-2*x* (shorter by only 0.3 mm, or ~ 7 %, Table 3.2), seems unlikely to have affected the efficiency of pollination. *Acacia auriculiformis* shared the same pollinators, and still set heavy seed crops with more seeds per pod than AM-4*x* (Fig. 3.7), despite *A. auriculiformis* style length being shorter than that for both *A. mangium* cytotypes.

The percentage of male flowers within a spike differed significantly among species/ploidy combination between years, with AM-4*x* having fewer male flowers than AM-2*x* in 2008 and significantly more in 2009 while AA-2*x* had the highest percentage in both seasons (Table 3.1). SEDGLEY *et al*. (1992d) reported *A. mangium* trees in Australia and Malaysia had somewhat higher percentages of male flowers, ranging from 3.5 to 88.3 % males, than *A. auriculiformis* trees, with 0.1 - 19.9 % males in individual trees. In *A. mearnsii*, the proportion of male flowers was higher (93 %) in years of heavy flowering than those of light flowering (52 %) and this was suggested to be associated with changes in water availability (ARONSON 1992; MONCUR *et al.* 1991).

Changes in the proportion of male flower per spike found in this study are unlikely to affect levels of seed production in *A. mangium*. Even for the highest percentages of male flowers observed in our study (23 %) the number of perfect flowers per spike was still much greater than the maximum number of pods that can

mature on a single spike, which is an average of 3.1 pods per spike with range of 1 - 12 pods (GRIFFIN *et al.* 2010). Furthermore, the ovule number for both cytotypes exceeded the average number of mature seeds per pod (Table 3.2 and Fig. 3.7). Hence, we conclude that neither variation in andromonoecy or ovule number is likely to create a mating barrier between tetraploid and diploid *A. mangium*.

The significantly larger diameter of both stigma and polyad of AM-4*x*, compared with those of AM-2*x* and AA-2*x* are in accord with the expectation of gigantism at higher levels of ploidy (LEVIN 1983). Microscopy confirmed that polyads of all species/ploidy combination contained 16 pollen grains, with eight pollen grains in the centre and eight pollen grains arranged singly on the periphery (Fig. 3.5). The relatively larger size of AM-4*x* polyad was therefore due to an increase in pollen grain size, rather than a change in number of pollen grains per polyad. However, the width of the stigma cup was significantly greater than that of polyad regardless of cytotype (Fig. 3.5). This enabled effective polyad-stigma combinations between cytotypes of *A. mangium* and between the two species. Once again we conclude that these morphological differences were not able to inhibit pollination. The considerable difference in size of polyad and stigma between cytotypes and species could result in a change in frequency of fruit pods with multiple paternities arising when two or more polyads are deposited on the stigma. Hence, the genetic structure of the seed crop might be modified. Pollination in acacias generally results from one polyad adhering to each stigma as reviewed by KENRICK (2003), with the resulting progeny being full sibs. The multiple-parent phenomenon was detected in *Acacia* hybrid with a small percentage of stigmas having two polyads deposited (SORNSATHAPORNKUL and OWENS 1998a;

SORNSATHAPORNKUL and OWENS 1998b) and in *A. melanoxylon*, with 0.08 - 0.15 % of tested pods in two different populations having more than one pollen parent (MUONA *et al.* 1991). Deposition of two polyads per stigma may happen in *A. mangium* and *A. auriculiformis* as well (SORNSATHAPORNKUL and OWENS 1998b), and the wider stigma of AM-4*x* may make this more likely in inter-cytotype pollination involving AM-4*x* pistils. Finally, the number of pollen grains per polyad was greater than the number of ovules per ovary, as is normal in *Acacia* species (KENRICK 2003; KENRICK and KNOX 1982) indicating that provided the pollen was of high viability a single polyad should be capable of effecting fertilization of all ovules.

3.4.3. Pollen quality

Pollen quality is critical for studying reproductive biology of *Acacia* since seeds in a pod are set following a single pollination event. However, *Acacia* pollen viability is known to have a short duration and loss of pollen quality may be due to ageing of flowers and environmental effects (KENRICK 1994). We used an *in vitro* test of fresh pollen from our three species/ploidy combination to determine if there were any substantial differences; although this test does not always reflect the true viability of pollen (SEDGLEY and GRIFFIN 1989; SEDGLEY and HARBARD 1993). SEDGLEY *et al*. (1992c) reported that there was an average of 4.6 pollen tubes in the styles of outcrossed AM-2*x* flowers holding a germinated polyad, whereas *in vitro* germination of polyads showed an average of only 1.5 pollen tubes per germinated polyad. The maturation of *Acacia* seed during the reproductive cycle was subject to pod abortion irrespective of the pollen quality. The relationship between pollen quality and seed set in controlled pollination of *A. myrtifolia* was studied by

KENRICK (1994). Pollen quality did have a significant effect on the number of pods set per flower pollinated at three weeks after pollination, but did not affect either the number of pods harvested or the number of seeds per pod.

The pollen of AM-4*x* may be slightly less fertile on average than that of AM-2*x* and AA-2*x*, although high clone-to-clone variation within cytotypes was apparent. However, the pollen viability of AM-4*x*, at 38.6 %, should be sufficient, unless other factors are involved, to produce open-pollinated 3*x* seeds from crosses between AM-2*x* and AM-4*x*. The smaller percentage of polyads with greater than three pollen tubes per polyad of AM-4*x* polyad (Table 3.3, Fig. 3.6) might have contributed to the lowernumber of seeds per pod observed in AM-4*x*, which were derived predominantly from selfing.

3.4.4. Insect visitors

Most *Acacia* species require pollen vectors to transfer pollen from the anther to the stigma for reproduction (SEDGLEY 1989b; SEDGLEY and GRIFFIN 1989). Understanding insect behaviour is therefore important when considering the likelihood of, and barriers to, inter-cytotype crossing in the Bau Bang seed orchard.

In our preliminary observations, floral visitors, mainly honeybees, seemed not to discriminate strongly between diploid and tetraploid of *A. mangium* as well as diploid *A. auriculiformis*.For most trees sampled, two or three of the three polyad types were found on the bee visitors (Table 3.4). However, honeybees may have been more attracted by AA-2*x*, rather than both AM-4*x* and AM-2*x*, because AA-2*x* polyads were present in all honeybee samples regardless of whether they were collected from *A. mangium* or *A. auriculiformis* trees. It appears that insect behaviour

is unlikely to create an absolute barrier to inter-cytotype crossing. However, the observations were made on only a small sample of trees, and a more comprehensive study would be required to reliably determine the proportions of intra- and inter-cytotype pollen movement in the orchard. A study on mixed-ploidy populations of *Chamerion angustifolium* showed that 73 % of all pollen came from within-cytotype pollinations, as a result of pollinator foraging behaviour (HUSBAND and SABARA 2004; KENNEDY *et al.* 2006). Similarly, in a natural population of *Heuchera grossularifolia*, some common visitors preferentially foraged one ploidy level more frequently and displayed a strong discrimination between diploid and autotetraploid plants; for instance, diploids were visited frequently by sweat bees and bumble bee workers, while tetraploids were visited by bee flies, moths and bumble bee queens (SEGRAVES and THOMPSON 1999).

Honeybees were the most common insect pollinator in this orchard, consistent with previous studies (SEDGLEY *et al.* 1992a; SORNSATHAPORNKUL and OWENS 1998b) and were also the most frequent visitors in the early morning (between 06h30 and 09h30). *Acacia mangium* and *A. auriculiformis* anthers dehisce early in the morning and honeybees are vulnerable to heat stress as the temperature grow hotter in mid morning (MARTINS 2004).

3.4.5. Yield of mature seed

Although the data on seed yield per pod is not directly relevant to the aim of examining the possible effects of phenology and flower morphology on mating patterns, it does contribute to understanding how the orchard is functioning as an interbreeding unit.

Like its Mimosoid relatives, *Acacia* was confirmed as having a low level of natural fruiting and high level of seed abortion within pods (KENRICK 1994; KENRICK 2003). The mean number of seed set per pod of AM-4*x* and AM-2*x* as well as AA-2*x* did not exceed nine; therefore, it was limited by the number of pollen tubes per polyad and the number of ovules per ovary. However, AM-4*x* produced less than half the number of filled seeds per pod than either of the diploid taxa in two consecutive years.

Although limited observation was made on floral visitors, the 98 % selfing rate in six of the AM-4*x* clones observed by GRIFFIN *et al.* (2012) appears unlikely to be due to lack of pollen movement between different AM-4*x* clones. AM-4*x* polyads were found on insect visitors to trees of all ploidy combinations, suggesting their widespread transfer between trees throughout the orchard.

The high level of outcrossing (97 %) reported by GRIFFIN *et al.* (2012) for AM-2*x* in the Bau Bang was consistent with previous studies. Natural populations of *A. mangium*, *A. auriculiformis* and *A. crassicarpa* were reported to have a high level of outcrossing based on isozyme analysis (MORAN *et al.* 1989). However, a comparison of outcrossing rates in a wider range of natural populations of *A. mangium*, BUTCHER *et al.* (2004) showed that there was great variation in outcrossing rate, ranging from complete selfing in populations with extremely low genetic diversity to complete outcrossing in the more genetically variable Papua New Guinea populations. Another study found that levels of outcrossing were high in four seed orchards of *A. mangium* in Vietnam in which flowering was heavy and synchronous, regardless of the orchard's natural provenance origin, whereas levels of selfing were high in two orchards where there was a low intensity of flowering and

flowering was asynchronous (HARWOOD *et al.* 2004). Thus, diploid *A. mangium* can display some degree of self-compatibility under some circumstances; however, this was not the case at Bau Bang.

Lower seed set per pod of the AM-4*x* trees is associated with high levels of observed selfing, which may be associated with a breakdown in the self-incompatibility system, under colchicine-induced polyploidy. PANDEY (1968) reported that colchicine itself affected the strength of self-incompatibility, even in individuals that did not increase in ploidy. As reported by KENRICK and KNOX (1989), in several breeding and hybridization experiments in diploid *A. mearnsii* and *A. decurrens* in South Africa, both *A. mearnsii* and *A. decurrens* set less seed per pod by self-pollination than cross-pollination.

The relationship between the sterility of ovules and seed production should also be considered. At the cellular level, BUYUKKARTAL (2008) investigated the causes for low seed set in the natural 4*x* *Trifolium pratense* L. (Fabaceae). The reasons for female sterility that led to 80 % of ovules being aborted in tetraploid *T. pratense* was confirmed as being a failure of megaspore mother cell formation and a defect in the early stages of meiosis or cell division (BUYUKKARTAL 2008). Such cytological effects might conceivably contribute to the lower seed set per pod observed in AM-4*x*, although overall fertility of the tetraploids remained high, with many thousands of viable seeds collected from each of the confirmed tetraploid clones in successive years of seed collection.

Phenology of flowering and differences in floral morphology seem insufficient to prevent inter-cytotype crosses in the Bau Bang orchard. Such crosses might fail because of incompatibility in pollen - pistil interactions, or cytological problems in

seed development due to imbalances in ploidy between the endosperm and embryo within seeds, which could result in a high level of seed abortion within pods (BURTON and HUSBAND 2000). Our own observations at Bau Bang leads us to expect that the post pollination interactions including imbalance of ploidy between endosperm and embryo within seeds could have a major effect on the genetic structure of the population of seeds at harvest time. Studies of this phase of the reproductive cycle are ongoing.

3.5. Conclusion

The phenophases of AM-4*x* and AM-2*x* was very similar. Although there were significant differences in some aspects of the morphology of spikes, flowers and polyads between tetraploid and diploid *A. mangium* as well as diploid *A. auriculiformis*, these differences were only small and did not appear sufficient to prevent crossing between AM-4*x* and the diploids of the two species. It is thus concluded that there were no barriers in phenophase or flower structure to inter-cytotype pollination of *A. mangium* and *A. auriculiformis*.

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Chapter 4

Pollen-pistil interactions between autotetraploid and diloid *Acacia mangium* and diploid *A. Auriculiformis*
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CHAPTER 5. POST-ZYGOCTIC REPRODUCTIVE BARRIERS

This chapter explores the possible mechanisms responsible for the differences in pod/filled seed yield amongst different types of inter-cytotype crosses and tetraploid outcrosses of *A. mangium* throughout the pod/seed development from fertilization to pod maturation.

In previous chapters, I have shown that neither differences in floral phenology and morphology between 4*x* and 2*x A. mangium* and *A. auriculiformis* nor foraging behaviour of the main insect pollinators (honeybees) between cytotypes, were strong enough to restrict inter-cytotype crosses within and between these two species (NGHIEM *et al.* 2011). Moreover, in controlled pollination experiments pre-zygotic reproductive barriers preventing inter-cytotype fertilization were not detected as pollen tubes were observed growing well in the style, entering the ovary, and penetrating the ovules regardless of mating type (NGHIEM *et al.* 2012). Therefore factors operating between fertilization and seed maturation must be limiting the production of viable triploid progeny.

5.1. Introduction

The difficulty of obtaining viable 3*x* progeny by reciprocal 2*x* x 4*x* crosses has been termed the "triploid block". This has been ascribed to the ploidy ratios (i) between the embryo, endosperm and maternal tissue that might differ from the ratio of 2:3:2 associated with normal seed development (LESTER and KANG 1998; RAMSEY and SCHEMSKE 1998); (ii) the actual ploidy ratio *i.e.* the ratio of maternal to paternal genomes (2:1) (the imprinting hypothesis) (HAIG and WESTOBY 1991; LIN

1984; MOFFETT 1956); or the effective ploidy *i.e.* a balance of qualitative genetic factors (Endosperm Balance Numbers hypothesis) (JOHNSTON *et al.* 1980; JOHNSTON and HANNEMAN 1999) in the endosperm that is critical for successful endosperm development and seed maturation.

The triploid block barriers may be incomplete in genera such as *Solamun*, *Citrus, Populus* and *Oenothera* where viable 3*x* progeny from either inter-cytotype crosses (2*x* x 4*x* and 4*x* x 2*x*) or unreduced gametes of (2*x* x 2*x*) crosses can be produced (DINU *et al.* 2005; GERACI *et al.* 1975; RAMSEY and SCHEMSKE 1998). There have not been any similar investigations in *A. mangium* and *A. auriculiformis*, as polyploidy has not been found in the natural populations of these species although 3*x* individuals of another species (*A. dealbata)* were observed at low frequency in a seed sample from a natural population (BLAKESLEY *et al*. 2002).

The phenomenon of abundant flowers and low yield of pod and seed is characteristic of mimosoid legumes including *Acacia* (KENRICK 2003). Typically, less than 1 % of open-pollinated flowers produce pods. MONCUR *et al.* (1991) reported 0.33 % for *A. mearnsii*, 0.62 % for *A. caven* in Chile and only 0.055 % for *A. tortilis* in Kenya (TYBIRK 1993). This may result from several possible causes of which the most common are pollen and/or pollinator limitation; competition for resources between individual pods; influence of environmental factors; and occurrence of mating incompatibility (FLEMING *et al.* 2007; KENRICK 2003; KNUD 1993; TYBIRK 1989; TYBIRK 1993; ZAKARIA and KAMIS 1991). In addition, since a flower is normally pollinated by only one polyad, the maximum number of *Acacia* seeds per pod is expected to equal the number of pollen grains per polyad. However,

fewer seeds than this number, per pod, have been reported in many *Acacia* species and other Leguminosae (KENRICK 2003; STEPHENSON 1992; TYBIRK 1997) as presumably not all grains germinate.

Fruit and/or seed abortion after reciprocal crossing between 2*x* and 4*x* plants has been reported for many taxa, such as *Vitis*, *Citrus*, and *Berberis* (EBADI *et al.* 2010; ESEN and SOOST 1973; HEO *et al.* 2007; HIRAMATSU *et al.* 2003; SUN *et al.* 2011; WAKANA *et al.* 2002; WAKANA *et al.* 2003). Recent investigations have documented that failure of fruit and/or seed development often commences at early stages of fruiting development (ARATHI 2011; SUN *et al.* 2011). ARATHI *et al.* (2011) discussed selective post-fertilization seed abortion due to maternal resource depletion or genetic load as possible mechanisms.

In this chapter, I assess the development and abortion, over time, of pods within spikes and seed within pods following inter-cytotype crosses within and between *A. mangium* and *A. auriculiformis* and tetraploid outcrosses of *A. mangium*, in comparison with open-pollination of the three parent categories. Differences in pod set for different positions within the spike and seed abortion rates for different positions within the pod, which might indicate patterns of competition of maternal resources, are also examined. Ovules from aborted and healthy pods of inter-cytotype crosses at 2, 5, and 7 weeks after pollination (WAP) were studied to find out if ovule growth within a pod is associated with pod abortion in early developmental stages. At maturity, the proportion of unfilled to filled seeds within a pod was examined for each mating type. The weight and size of unfilled and filled seed were also compared

and the parentage of the offspring of controlled crosses was checked to determine whether target crosses were achieved.

5.2. Materials and methods

5.2.1. Field trial and pollination treatments

This experiment was conducted in the hybridizing seed orchard planted in 2003 in southern Vietnam, which incorporates clones of AM-2*x*, AM-4*x* and AA-2*x*. This orchard was described in detail in Chapter 2.

Pollination treatments conducted for this experiment included: (i) controlled-pollination (CP: thinning of flowers within spikes, emasculating, applying target pollen, and bagged: at least 800 flowers/ cross); and (ii) open-pollination (OP: the spikes tagged and un-bagged: 200 - 500 spikes/ tree).

Table 5.1. Individual CP cross-combinations

Types of crosses	Clone IDs	Types of crosses	Clone IDs
AM-2*x* x AM-4*x*	82 x 11[1]	AM-4*x* x AM-2*x*	36 x 82[1]
	82 x 40		40 x 30
	68 x 66[1]		66 x 68[1]
	68 x 11		
	30 x 40		
AA-2*x* x AM-4*x*	6 x 11[1]	AM-4*x* x AA-2*x*	36 x 6
	156 x 36[1]		40 x 156
	156 x 66		
	84 x 66		
		AM-4*x* x AM-4*x*	36 x 11
			36 x 66
			36 x 40
			11 x 36

Note: [1] the cross-combinations from which young pod samples were collected

For the CP treatments, two types of cross-combination were manipulated: (i) inter-cytotype crosses within and between species; and (ii) tetraploid outcrosses between AM-4*x* trees (Table 5.1).

Intra-cytotype, intra- and inter-specific crosses of AM-2*x* and AA-2*x* were not repeated in this experiment because these crosses had already proved successful in terms of percentage of pods set and seeds per pod in the previous experiment (Table 4.4, Chapter 4).

The hand-pollination method applied in the CP treatment was described in detail in Chapter 2 following standard techniques (GRIFFIN *et al.* 2010; SEDGLEY *et al.* 1992b).

5.2.2. Field work

5.2.2.1 Young pod sampling

Pod samples were collected from two individual crosses from each of three different CP cross-combinations to investigate the development of ovules after fertilization (Table 5.1) at 2, 5, and 7 WAP. In the other twelve cross-combinations, for which available pods were limited, all were left on the tree to provide data on pod and seed development over time.

The pollination bags were removed 3 days after pollination and were replaced by muslin bags, to avoid the pollinated flowers being affected by increase of temperature and moisture in the bags or branch damage (Fig. 5.1). Aborted pods dropping inside the muslin bags were collected at 2, 3, 5 and 7 WAP and the remaining healthy pods were counted.

Three healthy pods from each of the crosses, together with 5 OP pods from the set of maternal spikes tagged at the same day that the CP crosses were manipulated, were sampled at the same time intervals.

All aborted and healthy pod samples were fixed in 3: 1 methanol: acetic acid solution for at least 24 h. The solution was replaced by 70 % ethanol for transportation.

Figure 5.1. Muslin bag used to collect aborted pods (AA-2*x* x AM-4*x* at 3 WAP) (scale bar = 5 mm)

5.2.2.2 Pod retention data record

The number of spikes and pods remaining alive was recorded at 2, 3, 5, 7 WAP and harvest (~ 18 weeks) for all CP crosses and the OP control spikes.

5.2.2.3. Pod distribution on the spike

The location of 4 - 5 week old pods on 15 to 20 open pollinated spikes was determined for three different clones of AM-2*x* and AM-4*x* and two clones for AA-2*x* (J.L.HARBARD unpublished data). The position of each pod on the spike was determined by measuring the distance from the proximal end of the spike to the site of pod attachment. The frequency (%) of pods attached at each position was calculated at 1 cm intervals along the spike length for each species.

5.2.2.4. Mature pod and seed harvest

The remaining CP pods were left for harvesting in late April - middle May (18 WAP). Ripening pods change from green to brown, stiff and dry. Each pod was harvested separately and labeled according to the cross-type and pollination tag. Total numbers of pods and seeds per pod were counted for every CP cross. The seeds extracted from each pod were categorized into two types: (i) filled seeds of small or normal size, and (ii) unfilled seeds of small or normal size (Fig. 4.1, Chapter 4). The position of filled seeds and unfilled and/or missing seed within each intact pod was recorded, and ranged from one to a maximum of 16, from the proximal end of the pod (closest to the peduncle) to the distal end (closest to the style). A similar record was made for OP parent pods/seeds as the control.

In order to acquire more data on the positions of filled and unfilled seeds after open pollination, an additional 20 intact mature OP pods were collected from 3 additional clones (different from the parent clones in Table 5.1) from each species/ploidy combination. These pods were picked at random from different branches for each parent category.

5.2.3. Laboratory work

5.2.3.1. Microscopy observation

To observe the development of ovules after fertilization inside the young pods, the clear-squash technique was used as described in Chapter 2 (MARTIN 1959; SEDGLEY *et al.* 1992c); however, times for softening and clearing pod samples in a sodium hydroxide solution (0.8N NaOH) were adjusted up to 15 - 18 min at 60^{o}C, instead of 8 - 9 min as had been used for flower samples.

Ovules within a pod were counted and measured under a fluorescence microscope (Zeiss Axioskop 2) with UV light at 200x magnification. Developing and undeveloped ovules were classified by size, measured as length and diameter (Fig. 5.7). The images were digitally captured by Axiovision 3.1 sofware (Zeiss, Germany).

5.2.3.2. Seed germination

The CP filled seeds harvested in two experiments, including diploid-crossed seeds of 2010 and inter-cytotype and tetraploid outcrossed seeds of 2011, were nicked and set for germination on moist filter paper, in petri-dishes at 23^{o}C, together with 30 OP seeds from three parent trees (10 seeds for each) of each AM-2*x*, AM-4*x*, and AA-2*x* as the controls.

The germinated seeds were sown in the glasshouse while DNA was rescued from the non-germinated seeds as described in Chapter 2.

5.2.3.3. Genotyping and ploidy determination

Microsatellite and flow cytometry methods were used to genotype and identify ploidy level for seeds or seedlings derived from each of cross-combination. These methods were described in detail in Chapter 2.

In order to decide whether individual progeny were really from the target crosses, the microsatellite results were classified into three categories: (i) cross confirmed - three to all six primers show that the progeny carries exact alleles from their parents (both mother and father); (ii) different fathers - more than one primer shows that the progeny carries an allele from a non-target father; and (iii) selfing – all six primers show that the progeny have only the mother's alleles (either homozygous or heterozygous)

5.2.4. Statistical analysis

Percentages of pod set for the pollinated flowers following each of the CP cross-types and for the tagged OP spikes were estimated for the weekly intervals (2, 3, 5, 7 and 18 WAP). However, these percentages needed to be adjusted for the individual crosses where three young pods were collected at 2, 3, 5 and 7 WAP to investigate ovule development (Table 5.1). Those pods were alive up to the point of harvest. Therefore, the proportion of pollinated flowers calculated to set the pods that were sampled (based on the ratio of sampled to total pods up to each harvest interval) had to be adjusted when calculating proportions of pod set for pollinated flowers at each time interval.

OP pod set per spike was calculated based on number of spikes tagged. For example, OP AM-2*x* gave a total 148 pods at harvest from 381 spikes (38.9 % spike fruiting). The 381 spikes were estimated to carry 82,677 flowers, based on an average of 217 flowers per spike (NGHIEM *et al.* 2011), thus 0.18 % of all flowers yielded a pod. Clearly only flowers with a normal gynoecium could set a pod. In an earlier study, I observed that, on average, only 77 % of the flowers were perfect (range 45.6 to 100 % for individual clones) - the rest being male. Initial flower counts were therefore adjusted by this factor before calculation of ratio of pod set to flowers treated, increasing the ratio of flowers to pods to 0.23% in the above example.

Analysis of variance was carried out on individual variates, including mean number of pods per spike, mean number of filled and unfilled seeds per pod, proportion and position effect of unfilled seeds within pod, and mean weight and size of filled and unfilled seed, using one-way analysis of variance (ANOVA). A square root transformation of the original data was applied where plots of residuals versus predicted values showed a non-normal distribution. Multiple mean comparisons were performed by Tukey-Kramer's range test at $\alpha = 0.05$. Analyses were conducted using SAS (version 9.2).

2 x 2 contingency χ^2 tests were also used to compare the proportion of undeveloped and normal ovules for inter-cytotype crosses *versus* open-pollinated parents.

A Seed Yield Index (SYI) was calculated as the number of filled seeds set per 100 pollinated flowers for all types of CP crosses and OP parents as well as individual cross-combinations of each CP cross-type.

5.3. Results

Early developmental stages of pods and ovules are illustrated in Figure 5.6 & 5.7.

5.3.1. Spike retention and pod set over time for open-pollinated and control-pollinated flowers

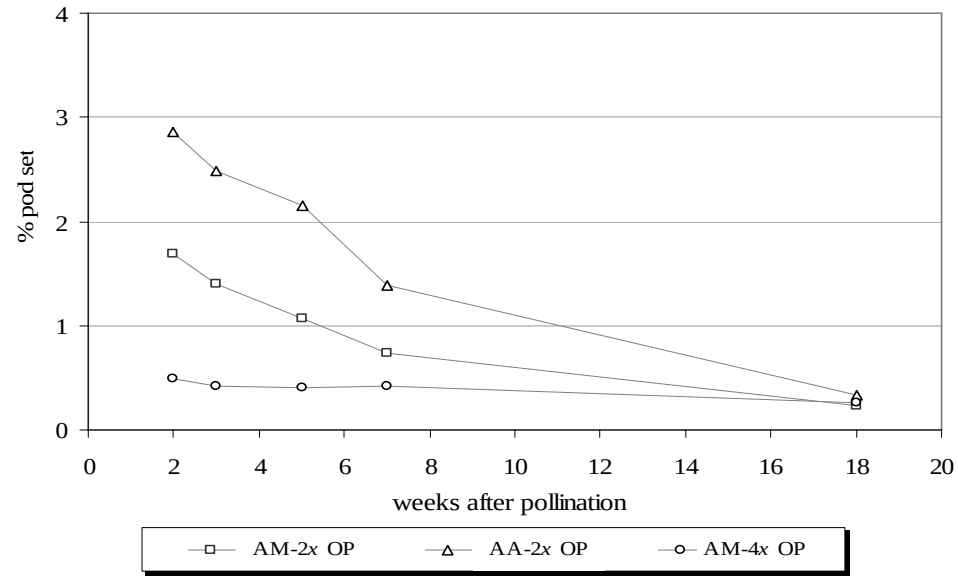
On the open pollinated (OP) controls most flowers had aborted by 2 WAP with less than 3 % developing into pods after 2 weeks and a further gradual decline at 3, 5, and 7 weeks. At 7 WAP pod set was 0.73 %, 1.39 %, and 0.41 % for AM-2*x*, AA-2*x* and AM-4*x*, respectively. However, all parent categories produced a similar proportion of mature pods, namely 0.23 %, 0.34 %, and 0.26 % (Fig. 5.2 and Table 5.2).

In a previous experiment, diploid crosses within and between species (*i.e.* AM-2*x* x AM-2*x*, AA-2*x* x AA-2*x* and AA-2*x* x AM-2*x*) produced more mature pods from the pollinated flowers (mean pod set exceeded 7 % - Table 4.4 Chapter 4) than either OP parents or inter-cytotype crosses. This is a measure of pod set potential where pollen limitation is completely removed and is considered as a control treatment in this experiment together with the OP data.

Following inter-cytotype control-pollination the initial pod set was higher than OP, but still less than 10 % of flowers developed into pods at 2 WAP for all cross-types. This percentage was somewhat higher in 4*x* x 2*x* crosses than in 2*x* x 4*x*

crosses in the first 3 weeks, and then dropped sharply from 5 weeks onwards for all these cross-types (Fig. 5.2 and Table 5.2).

(a)



(b)

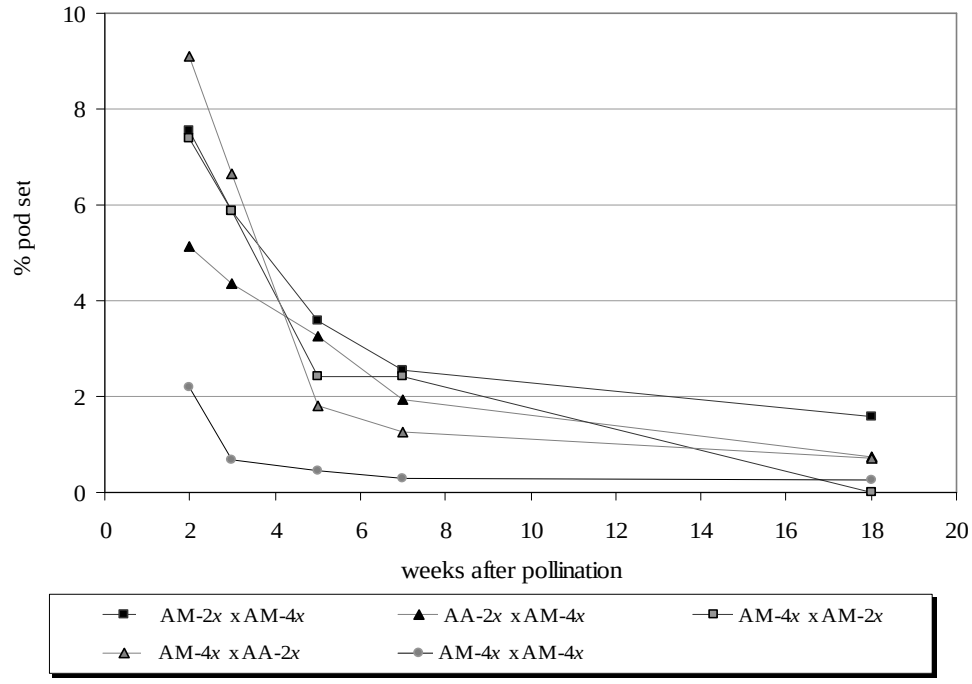


Figure 5.2. The mean percentage of pods retained with time for open-pollinated flowers (a) and control-pollinated flowers (b)

In the 2*x* x 4*x* cross-type, the initial pod set percentage was similar between and within species (1.94 and 2.54 %) at 7 weeks; however, the mature pod set was different at harvest time, with 1.57 % for the intra-specific crosses and 0.74 % for the hybrid (Fig. 5.2 and Table 5.2).

In contrast, in the 4*x* x 2*x* cross-type, inter-species showed slightly more successful initial pod set (9.08 and 6.65 %) than intra-species cross (7.40 and 5.87 %) at 2 and 3 weeks of pod development, respectively. Intra-species pod set remained unchanged between 5 and 7 week (2.43 %), but reduced to zero pod retention at harvest time, while inter-species declined to 1.82 % in 5 weeks but 0.71 % were still present at harvest (Fig. 5.2 and Table 5.2).

The percentage of AM-4*x* outcrossed pod set for the pollinated flowers was the lowest and reduced from 2.18 at 2 WAP to 0.26 % at harvest time (Fig. 5.2 and Table 5.2).

Since whole spikes aborted over time as well as individual pods within spikes, the mean number of pods per spike was calculated based on the number of spikes actually remaining at each assessment. After the initial drop the overall mean number of OP pods per spike remained unchanged from 2 WAP until harvest (4 – 5 pods/ spike) for AM-2*x* and reduced from 2 – 3 pods/ spike during 2 - 7 WAP to 1.7 pods/ spike at harvest for AA-2*x*. For AM-4*x* there was an average of 3.9 pods/ spike at 2 WAP and then there was a dramatic decline in AM-4*x* spike retention between 2 and 3 WAP, from 17.2 to 7.2 %, resulting in the mean number of pods per spike, for retained spikes, rising to 5.8 - 7.1 pods/ spike at 3 WAP to harvest. (Table 5.2).

Table 5.2. Spikes and pods retained at 2, 3, 5 and 7 weeks after pollination (WAP) until harvest, and mean pod percentage set per pollinated flower following inter-cytotype and tetraploid outcrosses and OP parents

Treatment	No. of spikes tagged	No. of flowers pollinated	Spikes retained (%)					Pods set [1] (%)					Number of pods/ spike				
			2 WAP	3 WAP	5 WAP	7 WAP	18 WAP	2 WAP	3 WAP	5 WAP	7 WAP	18 WAP	2 WAP	3 WAP	5 WAP	7 WAP	18 WAP
Inter-cytotype crosses																	
AM-2*x* x AM-4*x*	366	8,695	39.1	35.5	23.8	17.2	10.1	7.55	5.88	3.59	2.54	1.57	4.6	3.9	3.4	3.3	3.4
AA-2*x* x AM-4*x*	603	7,692	25.4	18.9	16.9	15.1	5.3	5.12	4.34	3.26	1.94	0.74	2.5	2.9	2.4	1.5	1.6
AM-4*x* x AM-2*x*	149	1,725	17.4	11.4	6.0	2.7	0.0	7.40	5.87	2.43	2.43	0.00	4.6	4.8	2.2	1.5	0.0
AM-4*x* x AA-2*x*	90	1,366	31.1	26.7	5.6	3.3	2.2	9.08	6.65	1.82	1.27	0.71	4.3	3.5	4.6	5.3	4.5
Tetraploid outcross																	
AM-4*x* x AM-4*x*	192	2,612	12.0	4.7	2.6	2.1	1.6	2.18	0.69	0.46	0.31	0.26	2.2	1.7	1.8	1.5	1.7
Open-pollinated parent trees (OP)																	
AM-2*x*	381	67,170	61.4	52.2	39.4	28.3	8.7	1.69	1.40	1.06	0.73	0.23	4.8	4.6	4.6	4.3	4.5
AA-2*x*	566	43,016	66.8	61.7	58.8	46.3	14.3	2.85	2.48	2.15	1.39	0.34	3.2	3.0	2.7	2.2	1.7
AM-4*x*	244	35,136	17.2	7.2	6.6	6.6	4.5	0.49	0.42	0.41	0.41	0.26	3.9	7.1	7.1	6.4	5.8

Note: [1] Pod set (%) = (total no. of pods harvested/ total no. of flowers pollinated after adjustment) x 100

The mean number of pods per spike varied amongst the CP cross-types depending upon whether *A. auriculiformis* or *A. mangium* was used as the mother. The mean number of pods per spike was almost unchanged from 2 WAP until harvest in AM-2*x* x AM-4*x* (3 - 5 pods/ spike), while this number reduced from 3 pods in early stages (2, 3, and 5 WAP) to less than 2 pods in 7 WAP and harvest for AA-2*x* x AM-4*x* (Table 5.2). In particular, an AM-4*x* spike was able to bear a variable number of pods following different types of cross, *i.e.* an AM-4*x* x AA-2*x* spike carried greater number of pods (3.5 – 5.3 pods/ spike) than did AM-4*x* x AM-2*x* spike (1.5 – 4.8 pods/ spike) while the AM-4*x* outcrossed pod per spike was the lowest and stable from initial to harvest stage (1.5 – 2.2 pod/ spike) (Table 5.2).

The distribution of 4 -5 week-old OP pods on spike was similar between *A. auriculiformis* and *A. mangium*, and between cytotypes (Fig. 5.3). Since about the first 1 cm of a spike does not carry any flowers (Fig. 3.8 Chapter 3), no pods were expected there. In all taxa the highest frequency of pod set was around the middle of the spike, at 5 - 6 cm, but some pods were present at all positions so there was no strong general position effect on the probability of a flower setting a pod at this stage (Fig. 5.3).

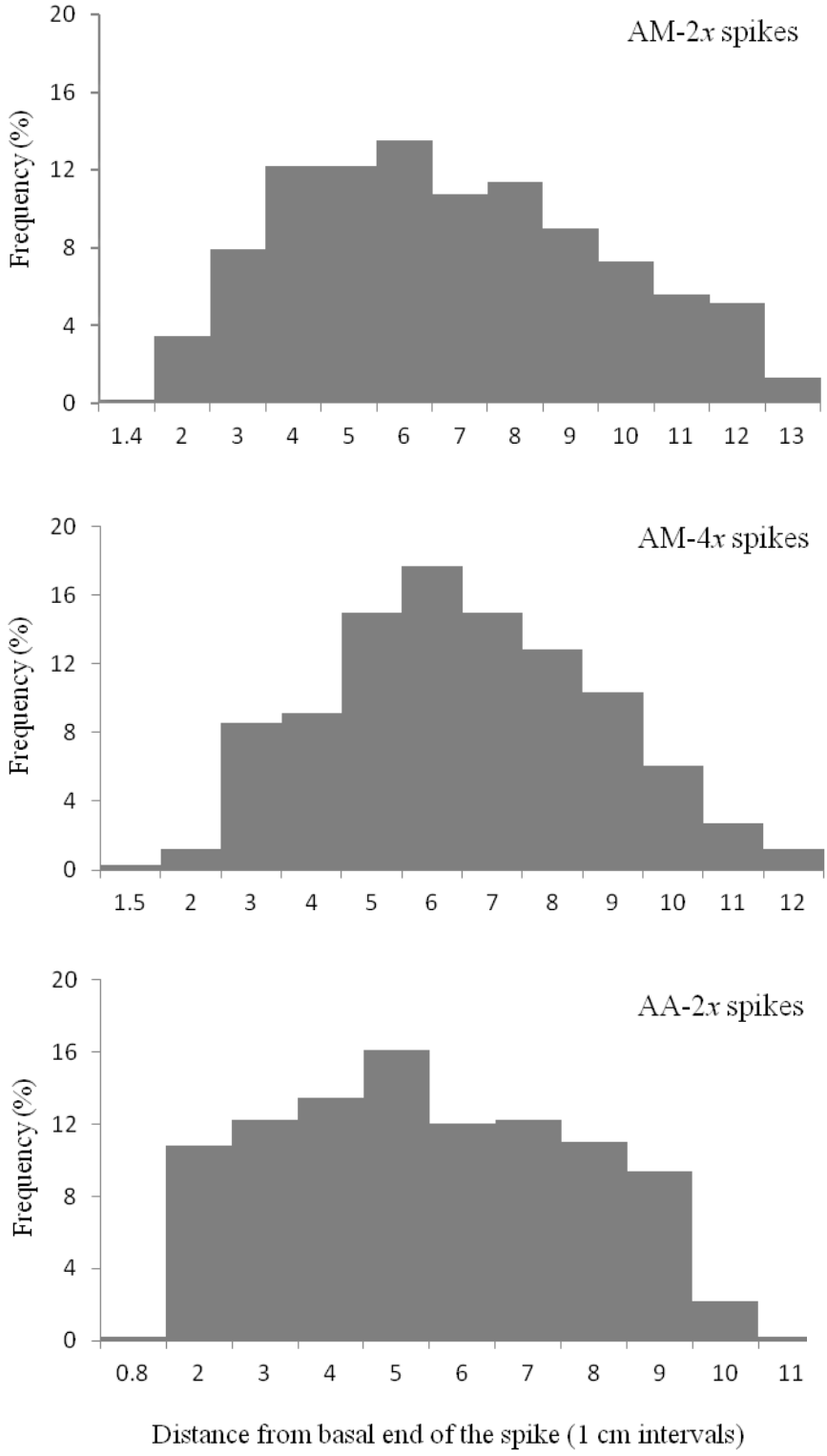


Figure 5.3. Frequency distributions of pod positions along the flower spike, for open-pollinated AM-2*x*, AM-4*x* and AA-2*x*.

(J.L. HARBARD unpublished data)

5.3.2. Pod and ovule development over time

Open-pollination pods of *A. mangium* elongated slowly between 2 and 7 WAP; from about 6 to 10 mm with no difference between 2*x* and 4*x*, but *A. auriculiformis* increased six fold in length from 6.5 mm at 2 WAP to 44 mm at 7 WAP (Table 5.3). A change in pod shape was observed from 5 weeks in *A. auriculiformis* with a flattening shape, while the *A. mangium* pods shape remained unchanged (Fig. 5.6).

A similar trend in both size and shape to healthy and aborted pods at the same time intervals was demonstrated depending on either *A. mangium* or *A. auriculiformis* was used as female in the CP crosses (Table 5.3). However, the pods aborted in the intervals before sample collection at 2, 5, and 7 weeks were always smaller than the healthy ones at the corresponding time (Table 5.3).

A total of more than 60 ovules were measured within OP pods of each parent (AM-2*x*, AM-4*x*, and AA-2*x*) and CP healthy pods of each inter-cytotype cross (AM-2*x* x AM-4*x*; AM-4*x* x AM-2*x*; and AA-2*x* x AM-4*x*) from the 2, 5, and 7 WAP collections. At 2 WAP, OP AM-2*x* ovules (54.7 µm) were smaller than both AM-4*x* and AA-2*x* ovules, which were similar in size (63.4 and 61.2 µm, respectively) (Fig. 5.4). However, OP AA-2*x* ovules had grown faster after 5 weeks post-pollination and reached an average size of 90.5 µm in length at the 7^{th} week, compared with OP AM-2*x* (64.8 µm) and AM-4*x* ovules (82.1 µm). The ovule size in the inter-cytotype crosses in early pod development stages was determined by the maternal parent (Fig. 5.4).

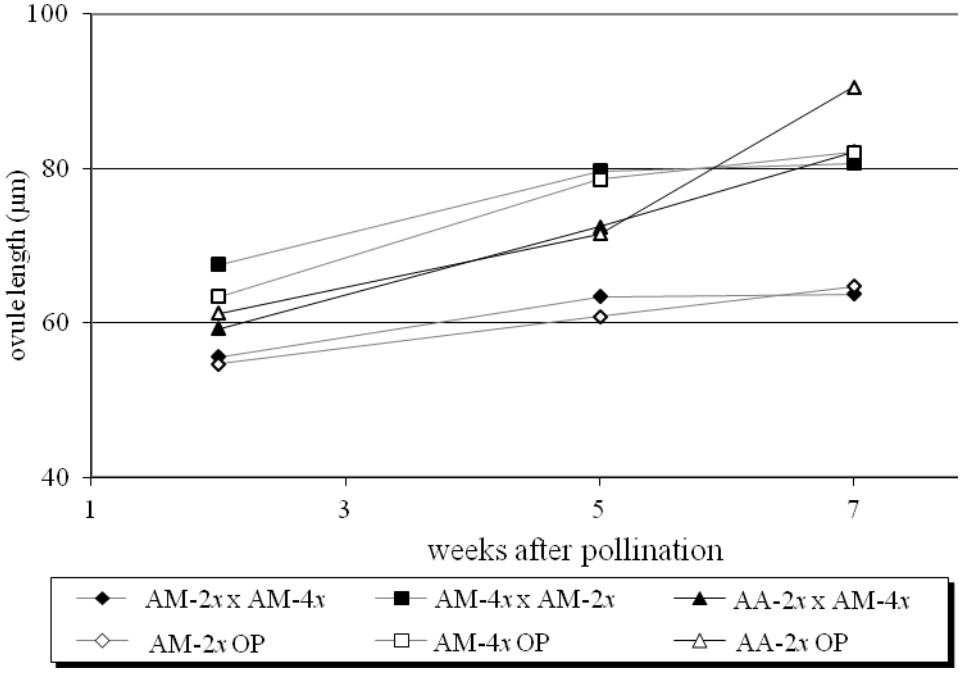


Figure 5.4. Ovule development following inter-cytotype crosses and OP parents

The number of ovules per pod across the 2, 5, and 7 WAP sampling dates averaged 12.8 across all taxa with a range of 9 to 16. There were slightly fewer ovules in aborted pods (12.4) than in healthy (13.1) and OP (12.9) pods (Table 5.3).

Variation in size among ovules became more obvious with time and I was able to classify for the 5 and 7 WAP ovules within pod into normal and undeveloped categories. Considering OP pods, the proportion of undeveloped ovules per pod ranged from 22.4 to 33.3 % for all parent categories. For CP pods from inter-cytotype crosses, this proportion was higher in AM-2*x* x AM-4*x* (ranging from 39.7 to 47.4 %) than in the reciprocal (21.9 – 29.3 %) and the range was wider in AA-2*x* x AM-4*x* (23.0 – 54.8 %) (Table 5.3).

Table 5.3. Pod development and mean number of developing seeds per pod showing proportion of undeveloped seeds per pod over time

Types ofcross	Young pod length (mm)									Mean no. of ovules/ pod[1]			Proportion of undeveloped ovules/ pod (%)					
	Aborted pod			Healthy pod			OP pod			Aborted pod	Healthy pod	OP pod	Aborted pod		Healthy pod		OP pod	
	2 ws	5 ws	7 ws	2 ws	5 ws	7 ws	2 ws	5 ws	7 ws				5 ws	7 ws	5 ws	7 ws	5 ws	7 ws
AM-2*x* x AM-4*x*	3.7	6.0	9.0	4.4	7.3	9.8	4.7	6.5	10.6	12.3	13.4	13.0	40.2	44.4	39.7	47.4	28.6	24.2
AM-4*x* x AM-2*x*	3.4	5.3	5.6	3.5	7.0	7.7	4.8	8.5	10.4	12.4	12.9	13.5	21.9	28.8	29.3	25.0	33.3	29.3
AA-2*x* x AM-4*x*	3.4	9.9	22.1	6.8	18.0	44.9	6.5	20.9	44.1	12.6	13.0	12.3	23.0	39.3	33.3	54.8	21.0	32.1

Note: [1] Average numbers of ovules in 2, 5, and 7 weeks (ws)-old pod samples before classification into normal and undeveloped ovules

The proportion of undeveloped ovules per pod was significantly higher in healthy inter-cytotype pods than in OP parent pods ($\chi^2_{(df=1)}$= 12.7, P < 0.001), but was not significant between aborted and healthy pods of inter-cytotype crosses ($\chi_{2(df=1)=}$ 2.96, P > 0.05) for 7 week samples. No significant differences were detected in the same paired comparisons for 5 week samples (Table 5.3).

5.3.3. Seed production and abortion following OP and types of cross

5.3.3.1. Mature seeds harvested following intra- and inter-cytotype crosses and comparison with OP.

The number of filled seed produced from 100 pollinated flowers showed considerable variable between intra- and inter-cytotype crosses, AM-4*x* outcross and OP parents (Table 5.4).

For OP flowers, there were only 1 to 2 filled seeds produced for every 100 flowers of AM-2*x* and AA-2*x* and less than 1 filled seed for AM-4*x*. These numbers were much lower than diploid crosses where 40 to 79 filled seeds were produced for every 100 hand-pollinated intra- or inter-specific flowers being. However, inter-cytotype crosses and the AM-4*x* outcross were the lowest with a mean range of 0.3 to 0.9 filled seeds for 100 inter-cytotype pollinated flowers and only 0.27 for 100 AM-4*x* outcrossed flowers (Table 5.4).

Table 5.4. Seed yield for intra- and inter-cytotype crosses, and OP parents

Treatments	No. of flowers pollinated	No. of filled seeds harvested	SYI[1]	No. of seeds/ pod		
				Filled	Unfilled	Total
CP types of cross						
Intra-cytotype outcrosses (2010)						
AM-2*x* x AM-2*x*	242	159	65.70	9.42 [a]	0.32 [c]	9.74
AA-2*x* x AA-2*x*	207	83	40.10	5.50 [bc]	1.35 [b]	6.85
AA-2*x* x AM-2*x*	259	204	78.76	8.85 [ab]	0.96 [bc]	9.81
AM-4*x* x AM-4*x*	1,112	3	0.27	0.60 [d]	4.40 [ab]	5.00
Inter-cytotype crosses (2011)						
AM-2*x* x AM-4*x*	8,695	76	0.87	0.80 [d]	5.83 [a]	6.63
AA-2*x* x AM-4*x*	7,692	23	0.30	0.47 [d]	5.06 [a]	5.54
AM-4*x* x AA-2*x*	1,366	9	0.66	0.95 [d]	3.80 [ab]	4.75
OP parent trees						
AM-2*x*	67,170	1,152	1.72	7.60 [ab]	0.68 [c]	8.28
AM-4*x*	35,136	224	0.64	2.85 [cd]	1.70 [b]	4.55
AA-2*x*	43,016	577	1.34	5.07 [bc]	1.14 [b]	6.20
Significant differences				***	**	n.s.

Note : n.s. = not significant, ** = $0.001 \leq P \leq 0.01$, and *** = $P \leq 0.001$. Letter for significant differences of cross-types (using Tukey-Kramer significant differences at $P < 0.05$)

[1] the number of filled seeds set per 100 pollinated flowers

Considering mean numbers of seeds set per pod, there were significant differences in filled seeds ($P \leq 0.001$) and unfilled seeds ($P \leq 0.01$), but not in total seeds per pod, amongst CP cross-types and OP parents (Table 5.4). For diploid inter- and intra-specific crosses and the OP all had a high proportion of filled seeds to total seeds within a pod. In contrast, all the inter-cytotype crosses and the AM-4*x* outcross produced less than one full seed per pod (Table 5.4). Across all such inter-cytotype

combinations only 10.7 % (108 out of 1010) of total seeds harvested were filled seeds (Table 5.4).

5.3.3.2. Distribution of aborted seeds within mature pods

Percentages of unfilled seeds within OP pods were less than 5 % for every position in AM-2*x*, but there was some suggestion of a linear trend reduced from 13% unfilled at the proximal to less than 5 % at the distal end of the pod in AA-2*x*. There was no trend within AM-4*x* pods, which had a higher proportion of unfilled seeds than either 2*x* and also fewer total seeds per pod (Fig. 5.5).

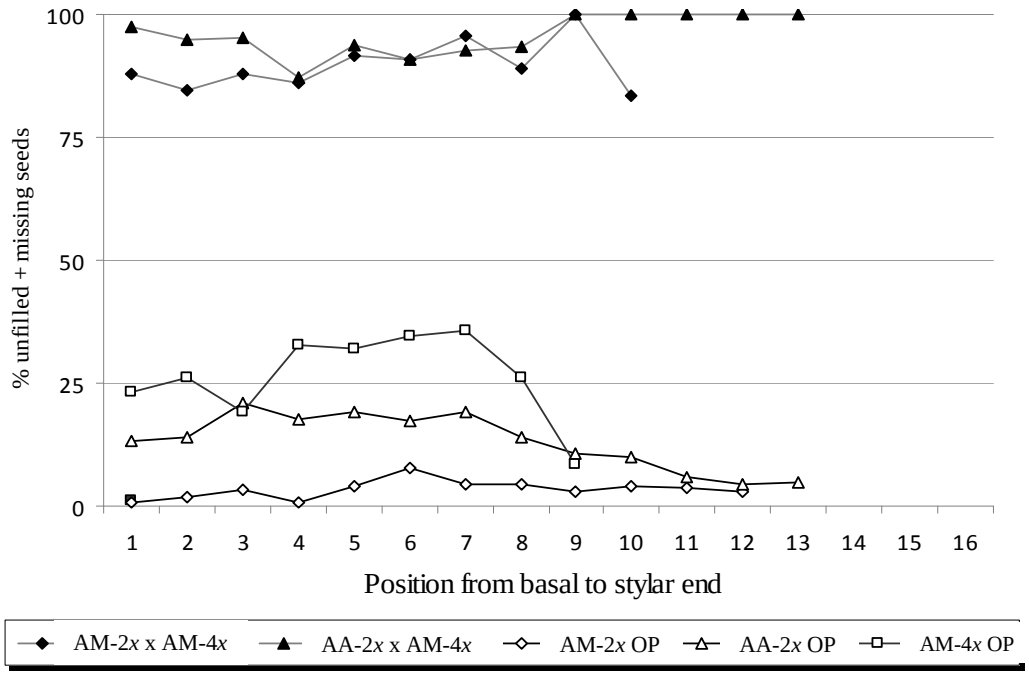


Figure 5.5. Frequency (%) of unfilled + missing seeds within pod

Since a high number of unfilled seed was observed within pods from inter-cytotype crosses, positions of these unfilled and/or missing seeds within healthy pods were examined. This was done only for the individual cross-combinations for which

at least 30 pods were harvested, so as to provide reasonable accuracy of estimation (Table 5.2.).

The 2*x* x 4*x* crosses within and between the two species yielded a maximum of 15 seeds per pod, similar to that for OP AM-2*x* and AA-2*x* pods. However, more than 75 % of 2*x* x 4*x* seeds harvested were unfilled and/or had missing seeds. Abortion of these inter-cytotype seeds occurred in every position within the pod, from the basal to the distal end (Fig. 5.5).

The mean unfilled seed proportion in a pod, regardless of position, varied between individual clones within parent categories. This proportion was significantly higher in 4*x* ($P \leq 0.001$) than in either 2*x* (Table 5.5).

Table 5.5 Mean proportion of seeds aborted within a pod following individual OP clones

Clone ID	Proportion (%)	Clone ID	Proportion (%)	Clone ID	Proportion (%)
AM-2*x*		**AA-2*x***		**AM-4*x***	
20	1.8	156	23.5	44	25.1
113	3.2	85	2.6	11	12.5
35	0.4	84	26.4	51	19.4
14	4.2	18	16.0	52	12.6
30	3.6	12	3.6	22	52.5
68	12.4	7	9.2	36	35.5
Mean	**2.4** [b]		**13.2** [b]		**26.4** [a]

Significant difference among three species/ cytotype combinations: $P \leq 0.0001$

5.3.3.3. Specific combination effects on mature seed harvest within the inter-cytotype crosses and AM-4x outcrosses

Variations in pod and seed yield for individual cross-combinations within the inter-cytotype crosses and AM-4*x* outcrosses are shown in Table 5.6. Within *A. mangium* the 2*x* x 4*x* cross-combinations succeeded in pod setting better than the reciprocal 4*x* x 2*x*. Three out of six 2*x* x 4*x* cross-combinations matured pods with a range of 0.24 to 2.82 % (Table 5.6), while none of the three combinations of six different genotypes for the 4*x* x 2*x* direction did . The number of filled seeds per 100 pollinated flowers (SYI) from the three AM-2*x* x AM-4*x* combinations which matured pods ranged from 0.24 to 1.62 (Table 5.6).

For the inter-specific combinations, some seed was harvested from both directions. All six AA-2*x* x AM-4*x* combinations produced at least one pod, with a range of percentage pod set from 0.05 to 4.79 % and the SYI from 0 to 1.06. The two AM-4*x* x AA-2*x* combinations both produced pods with a relatively similar SYI (0.53 – 0.71) (Table 5.6).

Three out of four cross-combinations of the AM-4*x* outcross failed to produce any pods and the remaining combination (36 x 40) set only 3 filled seeds giving a SYI of 0.27 (Table 5.6).

Evidently, there would be value in testing a wide range of specific combinations in attempting to breed triploids. For example, clone 82 of AM-2*x* was obtained the highest SYI in the cross-combinations with different male parents. However, the selected genotypes for the inter-cytotype hybrid did not exhibit much difference.

Table 5.6. Pod and seed harvested following individual inter-cytotype crosses and AM-4*x* outcrosses

Individual cross	No. of flowers pollinated	Pod set (%)	No. of seeds harvested		SYI[1]
			Filled	Unfilled	
AM-2*x* x AM-4*x*					
68 x 66	1,460	0.00	-	-	-
68 x 11	1,418	0.00	-	-	-
82 x 11	4,210	2.82	68	510	1.62
82 x 40	765	1.70	11	70	1.44
30 x 40	842	0.24	2	15	0.24
AA-2*x* x AM-4*x*					
6 x 11	1,215	1.16	3	44	0.25
6 x 66	752	4.79	8	198	1.06
156 x 36	3,570	0.81	13	112	0.36
156 x 66	1,081	1.57	8	104	0.74
84 x 66	1,826	0.05	0	2	0.00
AM-4*x* x AA-2*x*					
36 x 6	990	0.58	7	33	0.71
40 x 156	376	1.06	2	4	0.53
AM-4*x* x AM-4*x*					
36 x 11	376	0.00	0	0	-
11 x 36	541	0.00	0	0	-
36 x 66	583	0.00	0	0	-
36 x 40	1,112	0.45	3	22	0.27

Note: No pods matured from AM-4*x* x AM-2*x* crosses (see Table 5.1 and 5.2)

[1] the number of filled seeds set per 100 pollinated flowers

5.3.3.4. Mature seed weight, size and germinability

There was variation in weight and size of filled and unfilled seed among CP cross-types and OP parents; however, only the difference in weight of filled seeds

was significant ($P \leq 0.05$). The inter-cytotype seeds were smaller in size and weighed less than the OP parent seeds (Table 5.7).

Table 5.7. Seed parameters and germination

Cross types	Filled seed		Un-filled seed		Seed germination (%)
	Weight (mg)	Size (mm)	Weight (mg)	Size (mm)	
Controlled-pollination (CP)					
AM-2*x* x AM-4*x*	0.63 [b]	3.7/ 2.2	0.12	2.6/ 1.4	0.0
AA-2*x* x AM-4*x*	0.66 [b]	3.6/ 2.5	0.16	3.0/ 1.8	0.0
AM-4*x* x AA-2*x*	0.58 [b]	2.9/ 2.1	0.13	2.2/ 1.1	0.0
AM-4*x* x AM-4*x*	1.03 [ab]	4.1/ 2.4	0.24	3.1/ 1.7	0.0
Parent trees (OP)					
AM-2*x*	1.28 [ab]	4.2/ 2.8	0.24	3.6/ 2.0	100.0
AM-4*x*	1.51 [a]	4.4/ 3.0	0.24	3.5/ 2.1	96.7
AA-2*x*	1.55 [a]	4.6/ 3.5	0.22	3.9/ 2.4	96.7
Significant differences	*	n.s.	n.s.	n.s.	

All seeds from inter-cytotype and AM-4*x* outcrosses swelled, but did not germinate, while almost 100 % of OP seeds germinated under the same conditions (Table 5.7 and Fig. 5.8 b,c).

5.3.4. Genotype and ploidy identification

For the 2010 season crosses, the result of genotyping and flow cytometry on progeny of target crosses showed 100 % correct parental identity for the 36 intra-cytotype seedlings which survived. When DNA was extracted from 15 surviving seedlings from inter-cytotype crosses, 13 were shown to be contaminants by molecular testing (Table 5.8). However, 27 seeds of these inter-cytotype crosses did not germinate and so could not be tested. DNA was extracted from 7 out of 13 seeds that were dead 10 days after sowing in glasshouse, and 6 of these 7 were confirmed as triploids by flow cytometry (Table 5.8 and Fig. 5.8 d).

In 2011, DNA was extracted from 122 non-germinated seeds of the inter-cytotype crosses and 98.6 % those samples (68 out of 69) were confirmed arising from the target crosses (Table 5.8). Scoring alleles showed that 88.2 % (60 of 68 correct genotypes) carried three alleles at one or more locus and were confirmed as 3*x* individuals, while 11.8 % (8 out of 68) presented only two alleles at all loci tested although they also derived from those 2*x* x 4*x* crosses (Table 5.8).

Unfortunately, DNA could not be extracted from any of the AM-4x outcrossed seed.

Table 5.8. Genotyping for CP seeds harvested from the target crosses

Cross-types	No. of seeds tested	No. of seeds non germinated	No. of seeds death after 10 days	No. of seedlings survived	DNA extracted	No. of seedlings with			No. of 3*x* confirmed
						Cross confirmed	Different father	Self	
Intra-cytotype crosses (2010)									
AM-2*x* x AM-2*x*	17	0	4	13	13	13	0	0	-
AA-2*x* x AA-2*x*	15	2	0	13	13	13	0	0	-
AA-2*x* x AM-2*x*	22	5	7	10	10	10	0	0	-
AM-4*x* x AM-4*x*	3	3	-	-	0	-	-	-	-
Inter-cytotype crosses (2010 and 2011)									
2010									
AM-2*x* x AM-4*x*	26	16	5	5	5^{+}	0	4	1	3^{++}
AA-2*x* x AM-4*x*	29	11	8	10	8^{+}	0	2	6	3^{++}
2011									
AM-2*x* x AM-4*x*	81	82	-	-	35	34	1	0	30^{+++}
AA-2*x* x AM-4*x*	32	32	-	-	32	32	0	0	28^{+++}
AM-4*x* x AA-2*x*	9	9	-	-	2	2	0	0	2^{+++}

Note: One filled seed per pod was sowed for the 2*x* x 2*x* crosses, while all filled seeds harvested were sowed for the inter-cytotype crosses.

$^{(+)}$ 13 DNA progeny from inter-cytotype crosses were confirmed as diploids by flow cytometry; $^{(++)}$ using flow cytometry and $^{(+++)}$ using microsatellite markers

Figure 5.6. Inter-cytotype pods (scale bars= 2 mm)

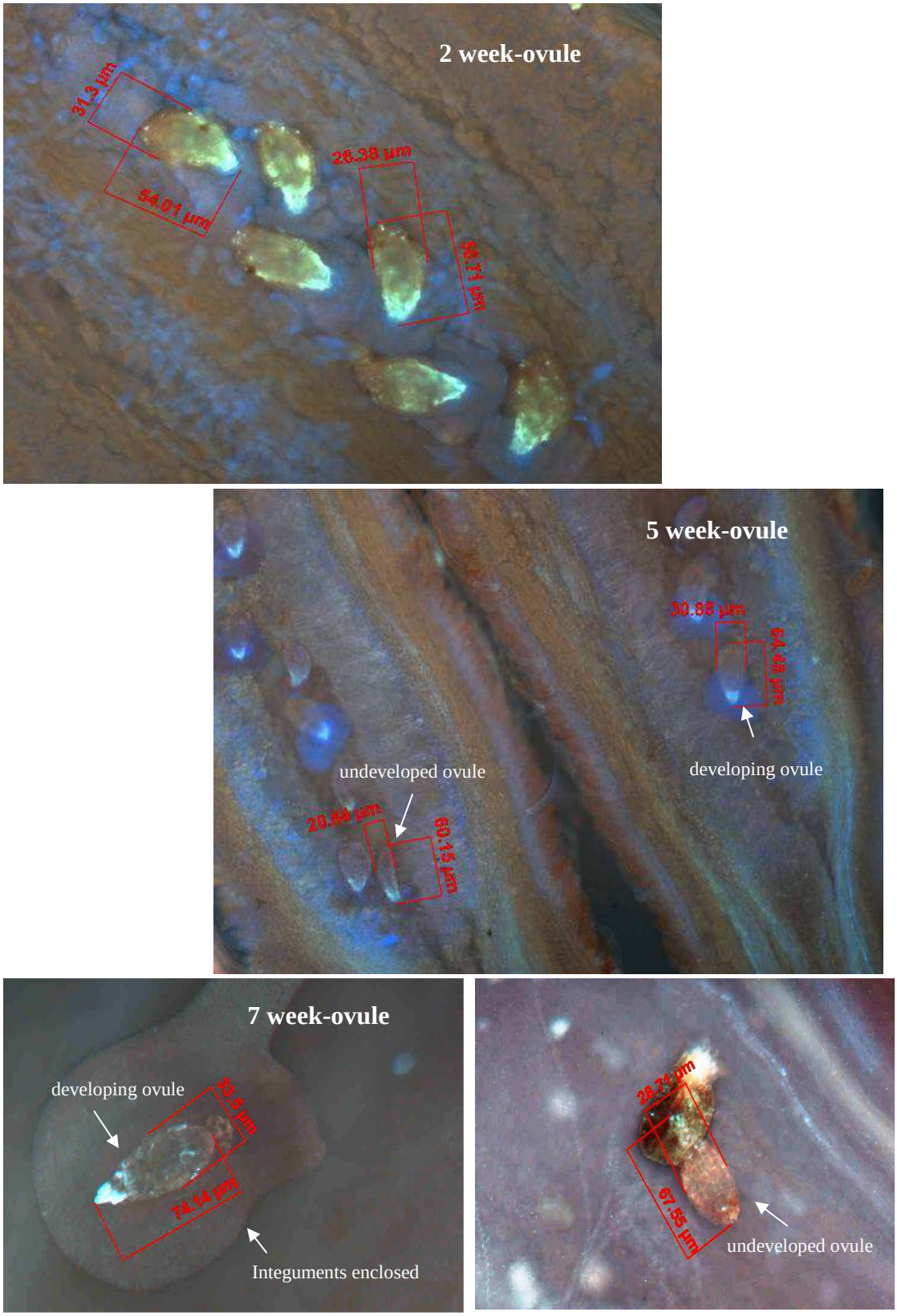


Figure 5.7. Ovule development after 2, 5 and 7 weeks post-pollination

Figure 5.8. CP pod and seed set (a); seed germination (b & c) and death (d) (scale bars = 5 mm)

5.4. Discussion

Following the reproductive phase of pollen - pistil interaction in angiosperms, excepting apomitic and parthenocarpic species, initial fruit development depends upon successful post-fertilization development. This process commences with growth of the nucellus and the integuments, and elongation of the embryo sac, resulting in increase in size of the fertilized ovules and then size of seeds and fruits. (SEDGLEY and GRIFFIN 1989). However, the probability of a particular fruit/ seed developing to maturity depends on a range of genetic and environmental factors.

In species such as *Acacia* it is normal to produce many more viable fertilized ovules than can be supported by the resources available from the maternal tree. There is therefore a process of competition between developing ovules for limited resources and the weaker ones will abort (ARATHI 2011). Since ovules/seeds are packaged within fruits and a whole fruit can abort, it is possible that this eliminates many seeds which are genetically quite capable of developing (KÄRKKÄINEN *et al.* 1999; STEPHENSON 1992; WESSELINGH 2007).

Genetic sources of variation in development are inbreeding, where deleterious recessive genes are expressed and cause mortality or at least reduced vigour and so reduced ability to compete for resources (CHARLESWORTH and WILLIS 2009; HARDER *et al.* 2012; HUSBAND and SCHEMSKE 1996), and various genic and chromosomal imbalances, examined in current comprehensive reviews (RAMSEY and SCHEMSKE 1998; RANNEY 2006). These latter factors are the most likely major cause of problems in the inter-cytotype crosses in this study. Abnormal endosperm development is a common problem with inter-cytotype crosses (ESEN and SOOST

1973; LESTER and KANG 1998; TAKAMURA and MIYAJIMA 1996; WAKANA *et al.* 2003) and can retard zygote development and result in seed abortion.

I have shown that the failure to produce pods and filled seeds from the inter-cytotype crosses between AM-2x, AA-2*x* and AM-4*x*, compared to the intra-cytotype crosses of AM-2*x* and AA-2*x* (Table 4.4 Chapter 4), did not result from pre-zygotic barriers (NGHIEM *et al.* 2012). The present study investigated how the pod and seed development in inter-cytotype and AM-4*x* controlled-outcrosses compared with the normal development after open-pollination. The results provide some insights into the causes of fruit abscission and seed abortion and are discussed in the following sections: (i) timelines for spike, pod and ovule/seed development; (ii) variation in seed yield and abortion with direction of inter-cytotype cross and specific cross-combinations; and (iii) reduced seed mass and viability of inter-cytotype crosses.

5.4.1. Timeline for spike, pod and ovule development

5.4.1.1. Spike and pod development over time

- Spike retention and pod set number per spike over time

In this part of the experiment, whole spikes were observed to abort over time. This was reflected in progressive reductions in spike retention and changes in the mean numbers of pods per spike over time (Table 5.2). TYBIRK (1993) stated that abortion during pod developing periods might be a response of limitations in resource availability for infructescences, branches and even whole trees. Moreover, the extent of spike and/or pod drop during development can be strongly affected by environmental factors, such as weather conditions and seed predation (KENRICK 2003; STEPHENSON 1992). For AM-2*x* and AA-2*x* parents, the spike retention at 7

WAP was about half of that at 2 WAP for both CP and OP. In contrast, the AM-4*x* mothers had already dropped a larger number of spikes in the OP by 2 WAP (only 17.2 % retained) and there was a further threefold reduction (6.6 %) by 7 WAP. Reductions were higher for the CP crosses (from 17.4 % to 2.7 % in AM-4*x* x AM-2*x*; 31.1 % to 3.3 % in AM-4*x* x AA-2*x*; and 12.2 % to 1.6 % in AM-4*x* x AM-4*x*) (Table 5.2). This showed that the effects of mating type begin to express early in the post-fertilization period. However, there were generally more OP spikes retained on the tree than CP spikes over time (2 to 7 WAP) (Table 5.2). This suggested that CP has had some effects on spike retention, most likely the impacts of bagging and handling; however, this did not affect the pod set (%) which was higher for CP in all cases.

Due to the loss of spikes, the mean number of pods per retained spike changed among CP crosses and OP parents over time. In general, OP spikes of *A. mangium*, regardless of cytotype, carried more pods (4 – 7 pods) than *A. auriculiformis* spikes (1 – 3 pods) (Table 5.2). The pod number per AM-2*x* spike was unchanged through the maturation period, while for AM-4*x* the few spikes that persisted carried a higher average pod number from 3 weeks onwards. The apparent increase in pods per AM-4*x* spike is not a real physical effect; it reflects the high rate of abscission of whole spikes many of which would have had below average numbers of pods per spike. This phenomenon might be relevant to the theory on formation of resource-sinks. STEPHENSON (1992) judged that pod development has the potential for attracting resources and act as resource sinks. In *A. auriculiformis*, OP pod number per spike reduced gradually to half at harvest (1.7 pods/ spike) compared with initial set (3.2 pods/ spike) (Table 5.2). The unreduced mean number of OP pods attached on a

spike of AM-2*x* and AM-4*x* trees from 3 weeks to harvest in my study was not similar to SORNSATHAPORNKUL and OWENS (1999) who reported that number of natural *Acacia* hybrid (*A. mangium* x *A. auriculiformis*) pods declined from 15 pods at 7 days and 4 – 12 pods after 44 days to only 2 pods per spike at 55 days and then remained stable to harvest.

There was a similar trend in inter-cytotype crosses where whole spikes also abscissed. The mean numbers of pods remaining on each spike was affected by the maternal taxon. There were less than 2 pods/ spike for AA2*x* x AM-4*x* crosses at harvest, 3 - 4 pods/ spike for AM-2*x* x AM-4*x* crosses, and variable numbers for intra- and inter-specific 4*x* x 2*x* crosses. AM-4*x* outcrosses displayed low spike and pod retention (Table 5.2).

- Pod retention over time

- OP pod set: Proportions of OP flowers setting pods also varied between the three species/cytotype parents (AM-2*x*, AM-4*x*, and AA-2*x*). The OP flowers dropped massively during the first 2 weeks after spikes were tagged, resulting in less than 3 % of initial pods being produced, namely 1.69 %, 0.49 %, and 2.85 % for AM-2*x*, AM-4*x*, and AA-2*x*. Pod production declined gradually over the next few weeks (3, 5, and 7 WAP) and finally was similar at harvest time with approximately 0.4 % (Fig. 5.2 and Table 5.2). The phenomenon of pod abscission during the first weeks of development was demonstrated in many *Acacia* species (TYBIRK 1989; TYBIRK 1993; TYBIRK 1997) and other perennial tree-legumes (ARATHI 2011). In Australian acacias, *e.g.* the natural *Acacia* hybrid and *A. suaveolens*, young pods were aborted massively during the first 8 weeks post-fertilization (SORNSATHAPORNKUL and OWENS 1999b; TYBIRK 1993). Less than 1 % of female

flowers producing mature pods after open pollination have been reported in many other *Acacia* species (KENRICK 2003; MONCUR *et al.* 1991; TYBIRK 1993). Interestingly, in this study all OP pod set converged on the same percentage at harvest even though levels were different early on. This is circumstantial evidence for resource limitation of a tree crown supporting a limited number of developing pods. Variation in pod set between individual trees within a population is expected (KENRICK 2003), and this was observed in my experiment. Pod set between the nine individual parents observed ranged from 0.03 to 0.58 % (data not presented).

- Intra-cytotype CP pod set: Mature pod harvest was higher in CP than OP, particularly for the diploid outcrosses within and between *A. mangium* and *A. auriculiformis*, which exceeded 7 % pod set on average (Table 4.4 Chapter 4). This is within the range reported in the literature. SEDGLEY *et al.* (1992b) recorded the pod set percentage of CP reciprocal inter-specific crosses of these two species between 0.0 to 20 % for 15 different individual combinations and KENRICK *et al.* (2003) found that mature pod set percentage of *A. mearnsii* increased from 1.3 % by open-pollination to 21.2 % by hand cross-pollination.

- Inter-cytotype CP pod set: Averaged across all cross types initial pod set at 2 WAP was substantially higher in CP (5.12 – 9.08 %) than for OP (0.49 – 2.85 %) (Fig. 5.2 and Table 5.2). Two major effects are probably involved. Firstly, under OP an unknown number of flowers would be pollinated to develop into pod. Secondly, the reduction in flower density on the spikes (number of flowers within spikes from an average of 94.4 for AA-2*x* and 180 - 217 for AM-4*x* and AM-2*x*, respectively, to 25 – 30 flowers before hand-pollination) as the CP spikes thinned could have reduced competition for resources between developing pods. However,

correspondingly more CP pods were dropped between 2 and 7 WAP so that the pod set percentages declined from 5.12 – 9.08 % at 2 WAP to 1.27 – 2.54 % at 7 WAP, and reduced further by time of harvest (18 WAP) (0.0 – 1.57 % pod set) for all CP cross types (Table 5.2).

Considering the direction of inter-cytotype cross, the pod set percentage in 4*x* x 2*x* crosses was higher than in 2*x* x 4*x* crosses in the first 2 and 3 weeks of pod development. This could be due to pollen from the 4*x* plant being less viable than from the 2*x* plant as shown by both *in vitro* and *in vivo* evidence (NGHIEM *et al.* 2012; NGHIEM *et al.* 2011). This would result in fewer fertilized ovules and therefore less initial pod set from the 4*x*-paternal crosses. However, the retained pod percentage also reduced over time for all combinations with pod set percentage at harvest of 0.0 - 1.57 % over all inter-cytotype crosses (Table 5.2). Hence, there are obviously other presumably genetic factors operating within the flowers that were pollinated effectively.

- Tetraploid outcross: Pod set for AM-4*x* CP outcrossed flowers was very low, only 2.18 % at 2 WAP and 0.26 % at harvest time, which was similar to the percentage of OP AM-4*x* pod set (primarily selfed) (GRIFFIN *et al.* 2012; VUONG 2009), but much lower than 2*x* CP outcrosses (exceeded 7 % on average) (Table 4.4 in Chapter 4). I found no other reports comparing the effects of self- and outcross-pollination on pod/seed production in the familae *Leguminosae*.

- Pod distribution on the spike

Some studies have found that fruit initiated early, and/or located close to the source of nutrients, sequester more resources than fruit initiated later and/or located

more distally (MEDRANO *et al.* 2000; STEPHENSON 1992). There are hypotheses about such effects on the distribution of fruit within developing infructescences such as non-uniform pollination and floral architecture effects (MEDRANO *et al.* 2000; STEPHENSON 1992; WESSELINGH 2007). However, in my study, I observed no strong position effect on the probability of a flower setting a pod at 4 - 5 WAP in any taxon. Perhaps small variations in development can affect the sink strength of particular pods in a situation where there is an upper limit to the number of developing pods which can be carried by a spike, I found a maximum of 7 pods per spike at 2 WAP. Resource limitation, if it operates, does not seem to favour any one position within the spike (for example, the basal end), since pods are distributed relatively uniformly along the spikes (Fig 5.3).

5.4.1.2. Pod and ovule/ seed development over time

The early development of the two species was different. The young pods grew and changed into a more flattened shape in *A. auriculiformis* for OP and inter-cytotype pod with AA female from 5 WAP, while *A. mangium* pods had not changed much in both size and shape for OP and CP with an AM mother irrespective of cytotypes (Table 5.3 and Fig. 5.6). This was consistent with pods of the natural hybrid of *A. mangium* x *auriculiformis*, which becomes flat after 8 weeks post-pollination (SORNSATHAPORNKUL and OWENS 1999b). The timeline for growth and development of pod is therefore taxon-specific.

Across all OP taxa and CP combinations, mean number of developing ovules within a pod was similar (12.8 ovules/ pod) among the pods when sampled at 2, 5, and 7 weeks, but abscised pods (12.4) had slightly fewer developing ovules than healthy (13.1) and OP pods (12.9) (Table 5.3). These numbers were essentially the

same as those observed in the flowers (averaged 13 - 14.2 for AM 4*x* and 2*x*, and 15.5 for AA) (NGHIEM *et al.* 2011). However, the mean number of mature seeds including filled and unfilled seeds obtained in a pod was substantially lower, ranging from 5 to 10 seeds, for all intra- and inter-cytotype pods as well as OP pods (Table 5.4). Some ovules probably failed to develop between 7 WAP and maturity, with differences among cytotypes and/or species.

Differences in ovule size was observable from 5 WAP although the bigger ovules of AM-4*x* compared with AM-2*x* is probably an expression of gigantism owing to increased ploidy *i.e.* polyad and stigma diameter (NGHIEM *et al.* 2011). This is consistent with a dormant period of the zygote which has been reported in many angiosperms (RAGHAVAN 1986; SEDGLEY and GRIFFIN 1989). In particular, the dormant period of the natural hybrid *A. mangium* x *A. auriculiformis* has been reported as 2 months during which time many fruit aborted (SORNSATHAPORNKUL and OWENS 1999b). According to SORNSATHAPORNKUL and OWENS (1999), one possible cause is delay in endosperm development which functions as a nutrient source for the zygote. The development of endosperm only occurred when the integuments completely enclose the nucellus and the nucellus degenerates. In our samples, the integuments were fully developed at 7 weeks for both inter-cytotype and OP parent ovules (Fig. 5.7). It appears that that the cellularization of the free nuclear endosperm and endosperm development is associated with increase in ovule size and coincides with pod size increase (Fig. 5.6 and 5.7).

The significantly higher proportions of undeveloped ovules in healthy inter-cytotype pods than in OP pods at 7 weeks (Table 5.3) suggests progressive abortion of inter-cytotype ovules within pods during pod development. The timing of abortion

will affect the size of undeveloped seeds in mature pods (Table 5.7). Hence, the significantly higher proportion of aborted ovules/ seeds and the smaller seed size in the inter-cytotype pods than in OP pods (Table 5.3 and 5.4) suggested that the progressive abortion of ovules/ seeds continued through the later stages of pod development and was probably affected by genic imbalance between the different cytotypes rather than resource competition.

5.4.2. Variation in seed yield and abortion with direction of inter-cytotype cross and specific cross-combinations

5.4.2.1. Seed yield and abortion following OP and inter-cytotype crossing

For OP AM-2*x* and AA-2*x*, the seed yield index (SYI) averaged 1 - 2 filled seeds, and was lower (0.6 filled seeds) in OP AM-4*x* (Table 5.4). Comparing SYI for OP and controlled pollination treatments is not straightforward since not all OP flowers will have been pollinated. In diploid acacias, BUTCHER *et al.* (2004) suggested that competition for maternal resource limitation is largely between pods, rather than between ovules/ seeds within a pod since all seeds within a pod are siblings which result from a single polyad pollination (KENRICK 1994; KENRICK and KNOX 1982). This means if the gametes are compatible there should be a correlation between survival of ovules within a pod *i.e.* if one seed is filled then others are likely to be. The high proportion of filled to total OP seeds per pod from the diploids in my study was consistent with this interpretation. I observed 7.6 filled/ 8.3 total seeds and 5.1 filled/ 6.2 total seeds for AM-2*x* and AA-2*x*, respectively (Table 5.4). However, for OP AM-4*x* the ratio was quite different with only about half of total seeds being filled (2.9/ 4.6) (Table 5.4).

Such high proportions of unfilled seeds in OP AM-4*x* pod were probably explained by irregularities occurring in the sequence of meiosis and mitoses. In *Acacia*, KENRICK and KNOX (1982) reported that the genetic basis of the polymorphism for polyad grain number (*e.g.* 4, 8, 16, or 32 pollen grains) is controlled by the number of mitoses of the sporogenous cells preceding meiosis and 16 grain-polyads is a result of two cell duplications by mitosis each leading to four genetically identical daughter cells that each undergo meiosis. Each meiosis allows recombination and gives four genetically unique haploid cells. So for the polyad as a whole there are 16 unique combinations of the paternal genotype. Therefore although the zygotes within the pod are full sibs they can still have different genotypes. In this mechanism both the genetic load and the genic imbalance from abnormal chromosome pairing can be different between individual zygotes in a pod and this might explain viability differences. Thus, in AM-4*x* genetic effects on seed development are probably more important than competition for resources between these different genotypes. As expected there was variation between individual AM-4*x* clones tested in my study; for example, clone 22 had a very high mean proportion of unfilled seed within a pod (52.5 %), while clone 11 had 12.5 % (Table 5.5). The reason for significantly lower number of filled seed in CP AM-4*x* outcrossed pods (0.6 filled/ 5.0 total) than OP AM-4*x* pods is not clear. It is probably due to specific clone effects since I only worked with two AM-4*x* maternal genotypes in the crosses. If I had conducted 4*x* selfs then the results may have been more in line with the OP as four of the six AM-4*x* clones that I studied (Table 5.5) were also investigated by GRIFFIN *et al.* (2012) who reported them to be predominantly selfing under open pollination. I assume that there was some levels of outcross 4*x* pollination since the trees flowered in synchrony (NGHIEM *et al.* 2011) and I have shown that there are no

problems with pollen tube growth and ovule penetration (NGHIEM *et al.* 2012). The low SYI and filled seeds per pod for AM-4*x* outcrosses must therefore be related to the genotypes of the developing ovules/seeds rather than intra-pod resource competition.

Under CP the SYI was much higher in both diploid intra- and inter-species crosses (40 – 79 filled seeds) than in inter-cytotype crosses or the AM-4*x* outcrosses (all less than 1 filled seed) (Table 5.4). Since the maternal resources available to all trees in the orchard can be assumed to be reasonably similar, and our CP technique ensures that pollen was not limiting, we can infer that the reasons for the differences are primarily genetic and associated with the changes in ploidy level. This conclusion also applies to the 4*x* outcross. The finding is similar to a study of *Citrus* where ESEN and SOOST (1973) reported that the number of seeds in fruit was normal, but the number of fully developed seeds was extremely low following 2*x* x 4*x* crosses. These authors suggested that the seed failure was attributable to defective endosperm development. Based on chromosome configuration at meiosis, numerous reviews summarized that endosperm failure from crossing experiments involving different cytotypes is often caused by a deviation from the 2 maternal: 1 paternal genomic ratio required for normal endosperm development. Following 2*x* x 4*x* and 4*x* x 2*x*, these ratios are 2m:2p and 4m:1p, respectively both of which result in abnormal endosperm development (LESTER and KANG 1998; MOFFETT 1956; RAMSEY and SCHEMSKE 1998; RANNEY 2006; STIFT *et al.* 2010). As the endosperm has a function in nutrition and regulation of embryo differentiation (GRINI *et al.* 2002), the presence of a significantly higher proportion of undeveloped ovules in an early stage (5 and 7 WAP) and unfilled seeds in a mature stage in the inter-cytotype pods compared with

OP pods (Table 5.3 and 5.4) was proabably due to early degeneration or abnormality of endosperm development, which resulted in either embryo starvation or irregular embryo transition. Note that this does not explain the poor seed yield and high seed abortion in both AM-4*x* outcrosses and OP which was inferred that direct cytogenesis (*i.e.* chromosome behavior) affects the zygote development.

The effect of cross-direction was shown in the reciprocal of intra-specific crosses (AM-2*x* x AM-4*x vers.* AM-4*x* x AM-2*x*) but was not so obvious in the inter-specific crosses (AA-2*x* x AM-4*x vers.* AM-4*x* x AA-2*x*) (Table 5.4 and 5.6). In diploid, hybridization between these two species showed that *A. auriculiformis* used as female parent was more fertile than *A. mangium* (NGHIEM *et al.* 2012; SEDGLEY *et al.* 1992c). Thus, I need to work with more cross-combinations within each type of inter-cytotype cross to achieve a firm conclusion about the cross-direction effect. Moreover, in other *Leguminosae* (*i.e. Pisum sativum*) diploid female parents were produced more seeds than tetraploid females (CONICELLA and ERRICO 1993). These authors explained that the maternal/paternal genome ratio (2:2) in endosperm of 2*x* x 4*x* crosses is closer to the normal 2m:1p ratio and the endosperm develops faster and supports the embryo longer than the endosperm of 4*x* x 2*x* cross that has a 4m:1p ratio. Therefore, detailed embryological studies are warranted to describe the developmental stages of the embryo, endosperm, and ovular tissues in relation to seed and fruit morphology and abortion, as SORNSATHAPORNKUL and OWENS (1999) did for diploid *Acacia* hybrids.

5.4.2.2. Cross-direction and individual genotype effects on filled seed set

The influence of cross-direction can also be examined through individual cross-combinations from each of inter-cytotype cross-types (Table 5.6). Considering

the inter-cytotype crosses within *A. mangium*, three out of five individual combinations of AM-2*x* x AM-4*x* gave 0.24 – 2.82 % pod set and 0.24 – 1.62 filled seeds for every 100 pollinated flowers (SYI) and was more successful than the reverse (zero pods/ seeds set) (Table 5.6). Two (82 x 11 and 82 x 40) out of three combinations of AM-2*x* x AM-4*x* yielded the highest amounts of filled seeds. This supports the finding of some other studies who reported that 4*x* pollen sired more seed than 2*x* pollen in *Chamerion angustifolium* (*e.g.* HUSBAND *et al.* 2002) and in *Pisum sativum* (CONICELLA and ERRICO 1993). In *Populus* and *Oenothera* viable 3*x* seeds were also obtained more easily by 2*x* x 4*x* crosses (RAMSEY and SCHEMSKE 1998).

For the inter-cytotype hybrid in this study, two individual combinations of AM-4*x* x AA-2*x* had a similar number of filled seeds (0.53 and 0.71) for 100 pollinated flowers while six combinations of AA-2*x* x AM-4*x* had a range of 0.00 – 1.06 filled seeds (Table 5.5). At the pollen-pistil interaction phase, I found that for pollen tube growth and ovule penetration the AA-2*x* x AM-4*x* cross was the more promising direction (NGHIEM *et al.* 2012). Thus, the wide range of SYI among six individual combinations of AA-2*x* x AM-4*x* suggests that a further investigation with more parental genotypes in true reciprocal combinations is required.

In addition, for allopolyploids, an alternative hypothesis, endosperm balanced number (EBN) was proposed (JOHNSTON *et al.* 1980). According to JOHNSTON and HANNEMAN (1999), the endosperm failure in reciprocal inter-specific 2*x* x 4*x* crosses might depend on the qualitative differences between the chromosomes of the male and female, rather than the numerical ploidies that regulate normal endosperm development. Thus, if there is no genetic incompatibility between the two species

there could be more chance to achieve viable and useful 3*x* progeny from inter-cytotype hybrids than within the same species. In fact the three viable 3*x* genotypes from the screened 758 OP seeds harvested in this orchard were all derived from AA-2*x* mother trees (J.L. HARBARD unpublished). Furthermore, in a comprehensive review on the rate of polyploid formation in flowering plants RAMSEY and SCHEMSKE (1998) documented that allopolyploids are much more frequent than autopolyploids in nature.

5.4.2.3. Seed abortion position within a pod

In OP pods, the percentage of unfilled seeds was slightly higher in the proximal (~ 13 %) than in distal end of pod (~ 4 %) for AA-2*x*, but was low at all positions for AM-2*x* pods (0.8 - 3 %) and slightly higher for AM-4*x* pods (8.3 – 35.7 %) (Fig. 5.5). These results suggest that there are no obvious position effects on seed abortion in OP AM-2*x* and AA-2*x* pods and therefore no evidence of positional competitive advantage with respect to pollen tube growth or the maternal resources within the ovary that could affect ovule and seed development. This is at odds with the suggestion of TYBIRK (1997) who concluded that for the Africa acacias that he studied there was more maturation of the stylar embryos into seeds than those in more basal position, explained by competition between vigorously growing pollen tubes, leading to the formation of resource-sinks. Considering competition for resources, SUSKO (2006) proposed that fertilized ovules/ seeds located nearest to the peduncle, which supplies nutrients, water, and photosynthate, might have greater opportunity of maturing than ovules located further. However, in OP AM-4*x* pods the distribution of unfilled seeds was more or less uniform (Fig. 5.5) resulting from cytogenetic imbalance in 4*x* gametes.

In 2*x* x 4*x* crosses within and between species, the majority (more than 75%) of seeds remained undeveloped at maturity and there were no strong position effects in their location (Fig. 5.5). This suggests that abortion is more related to the particular genotype of the embryo rather than a response to some maternally controlled resource availability gradient.

5.4.3. Seed weight and viability

A significant reduction in seed weight was observed when the inter-cytotype and tetraploid outcross seeds, both filled and unfilled, were compared with those of OP parent seeds (Table 5.6).

For inter-cytotype seeds this strongly suggested that the embryos might be partially developed compared with perfectly developed seeds of open-pollinated parents. Embryoless seeds occurred in inter-cytotype crosses of grape and grapefruit (ESEN and SOOST 1973; WAKANA *et al.* 2002). This phenomenon is often postulated as a consequence of irregular and/ or early degeneration of endosperm which resulted in failure to regulate embryo differentiation at the globular stage to form the two cotyledon primordia which are very important for seed development (SORNSATHAPORNKUL and OWENS 1999b).

Variable seed sizes among the inter-cytotype crosses and AM-4*x* outcrosses in this study might result from difference in the time of endosperm degeneration and of cytogenic imbalance in the embryo that happens in the AM-4*x* outcrosses.

The few filled seeds obtained from the inter-cytotype crosses and AM-4*x* outcrosses only swelled and did not germinate whereas almost 100 % of the open-pollinated seeds did (Table 5.6 and Fig.5.8 b,c & d). This indicated that the

endosperm of these seeds may pass a critical development stage enabling embryogenesis to proceed, but it could still be irregular or incomplete. Alternatively, different genes expressed in the germination process may have led to seeds not functioning normally. As a result there was no germination and no viable offspring were produced.

5.4.4. Offspring genotype and cytotype determination

In 2010, DNA rescued from 6 out of 7 dead seeds after 10 day-sowing from inter-cytotype crosses within and between species were confirmed to be 3*x* by flow cytometry. In 2011, DNA obtained from 60 out of 69 ungerminated seeds was confirmed arising from the target crosses by microsatellites and almost all of them were 3*x* seeds, except for eight out of 68 progeny. These 8 progenies were presented only two alleles at all loci tested which are supposed to be diploids although they were also raised from those 2*x* x 4*x* crosses (Table 5.7). This might be related to chromosome behavior which is always considered to be cytogenetically complex in polyploids because of the occurrence of multivalent pairing. In their review, RAMSEY and SCHEMSKE (2002) stated that there is a high frequency of aneuploidy, including hypo- and hyper-euploids, in the gametes of neopolyploids.

5.5. Conclusion

The spike/ pod abscission over time in both OP and CP crosses was expected; however, the abortion in the CP inter-cytotype crosses and tetraploid outcrosses was likely to be correlated with genetic factors, rather than limited resource competition, and could take place within and between pods.

Genic imbalance between different ploidies (2*x* and 4*x*) is suggested to be the main mechanism responsible for significantly lower SYI in CP inter-cytotype crosses than in CP diploid crosses as well as OP parents, whereas the low SYI in 4*x* OP and CP outcrosses was probably related to both lethal genes and nonviable chromosomal rearrangements during mitotic processes operating between individual zygotes in a pod. Therefore, chromosomal/ genic imbalance or misappropriation of the paternal and maternal genomes probably resulted in the endosperm depletion or malfunction in the inter-cytotype crosses (AM-2*x* x AM-4*x*, AA-2*x* x AM-4*x* and their reciprocals as well as AM-4*x* outcross). Smaller and lighter 3*x* and 4*x* outcrossed seeds were produced, compared with the parent OP seeds. Triploid seed non-germination or non-viability was due to the embryo starvation or failure/ incompleteness of metabolic process at globular stage or lethal genes expression in the germination process. However, there were differences among the few cross-combinations of each cross-type studied, so testing a wider range of crosses increases the chance of getting crosses that work well.

In conclusion, although seed development remained a problem, hybridization between 4*x* and 2*x* for producing viable 3*x* offspring of these tropical *Acacia* species is still possible.

CHAPTER 6. GENERAL CONCLUSIONS

Development of infertile triploid (3*x*) lines by crossing tetraploids (4*x*) with diploids (2*x*) has been advocated as one of the potential approaches to reduce fertility of acacia species, including *Acacia mangium* and *A. auriculiformis*, which have been classified as invasive (GRIFFIN *et al.* 2011; KOTILUOTO *et al.* 2009; RICHARDSON *et al.* 2011; TURNBULL *et al.* 1998). The development of seedless clones for these tropical *Acacia* species, and their inter-specific hybrid is desirable, as they play an important role in short-rotation production plantations in Southeast Asian countries (MIDGLEY and TURNBULL 2003).

The work undertaken in this study has contributed to the understanding of the reproductive behaviour of diploid and tetraploid cytotypes of *A. mangium* and diploid *A. auriculiformis*. By examining the reproductive phenology and floral morphology of the different cytotypes and species, and the results of crossing among them, the reproductive barriers limiting the production of triploid (3*x*) progeny within and between the two species were identified.

My study was carried out in a hybridizing seed orchard in southern Vietnam, which incorporated clones of artificial auto-tetraploid *A. mangium* (AM-4*x*), diploid of *A. mangium* (AM-2*x*) and diploid *A. auriculiformis* (AA-2*x*). The stages in reproduction from flowering through to seed production were systematically examined (Fig. 6.1), as detailed below.

6.1. Phenology

The phenological investigation indicated no cytotype effect on the phenophase of *A. mangium*. Clones of AM-2*x* and AM-4*x* had a one-month flowering peak, in November - December, slightly earlier than the December - January peak for AA-2*x*. This is consistent with several studies of the flowering times of diploid *A. mangium* and *A. auriculiformis* in other Southeast Asian countries which reported that *A. auriculiformis* typically flowers later than *A. mangium* (JIWARAWAT *et al.* 1996; ZAKARIA and KAMIS 1992). Pods and seeds matured in April – May, over an 18-week period following anthesis (NGHIEM *et al.* 2011) (Fig. 6.1).

6.2. Floral morphology

The floral structure of AM-4*x* was similar to that of AM-2*x* and AA-2*x*, with both hermaphrodite and staminate flowers arranged along the flower spike. However, AM-4*x* had significantly shorter spike length than AM-2*x*, and significantly fewer flowers per spike. The AA-2*x* spike was the shortest and had the smallest number of flowers per spike. The proportion of male to hermaphrodite flowers within the spike was similar (less than 23 %) for all three species/ploidy combinations but differed significantly between years (NGHIEM *et al.* 2011). ARONSON (1992) reported changes in the proportion of male flowers per spike in *A. mearnsii* between years of heavy and light flowering density, and suggested it was associated with changes in water availability. AM-4*x* flowers had shorter styles, but the stigma and polyad diameters were greater than those of AM-2*x* and AA-2*x*. However, the width of the stigma cup was always significantly greater than that of individual polyads for all cytotype and species combinations. This strongly suggested no barrier to polyad-

stigma combinations between cytotypes of *A. mangium* and between the two species (NGHIEM *et al.* 2011) (Fig. 6.1).

6.3. Pollen visitors

A preliminary investigation of foraging behaviour by floral visitors, mainly honeybees, suggested that there was no strong discrimination by insect visitors between different cytotypes of *A. mangium* or between the two species (NGHIEM *et al.* 2011). This finding did not accord with studies of the pollination ecology of natural mixed-ploidy populations of other plant genera, for example *Chamerion angustifolium* and *Heuchera grossularifolia*, where pollinators and their foraging behaviour showed strong discrimination between different ploidy levels, associated with major ploidy effects on floral traits such as flower size and shape (HUSBAND and SABARA 2004; KENNEDY *et al.* 2006; SEGRAVES and THOMPSON 1999). Those variations in floral traits appear much more substantial than I have observed in the present study of *Acacia*.

In summary, although in other plant genera, different ploidy levels are often reproductively isolated by differences in morphological and ecological characteristics (LEVIN 1975; LEVIN 1983; LOWRY *et al.* 2008; RAMSEY and SCHEMSKE 1998; RIESEBERG and WILLIS 2007) in the current study I found no evidence that the floral phenology and morphology of the species/ploidy combinations or the pollinator foraging behaviour created reproductive barriers to inter-cytotype and inter-species pollination.

6.4. Seed yields and breeding systems under open pollination

Pod and seed yield did vary significantly amongst open-pollinated (OP) AM-2*x*, AM-4*x*, and AA-2*x* with means of 8 - 10, 4 - 5 and 7 - 8 seeds per pod, respectively (NGHIEM *et al.* 2011). Although I can postulate that all species/cytotypes will have cross pollinated to some extent, it is clear that not all are successful in producing outcrossed seeds. There is evidence of a deficiency in mature seed from 4*x* outcrosses and all inter-cytotype crosses. GRIFFIN *et al.* (2012) found that all OP seed from six AM-4*x* mothers was all 4*x* and that 98 % resulted from self pollination. All seeds sampled from eight AM-2*x* mothers was 2*x* and ~ 97 % outcrossed (this study did not distinguish whether the pollen parents were AM or AA). In an associated study the very low yield of viable seeds from inter-cytotype crosses was confirmed by J.L. HARBARD (unpubl.) who identified only three 3*x* progeny genotypes after screening 758 open-pollinated seedlings from the orchard by flow cytometry.

6.5. Pollen-pistil interaction in relation to subsequent seed yields

To understand whether the lower seed numbers in AM-4*x* pods and the extremely low OP 3*x* yield in the orchard was due to pre- or post-zygotic factors, I next investigated the pollen-pistil interaction phase of the reproductive process for the five possible types of mating combinations that could occur in the orchard: (i) selfing of each of AM-4*x*, AM-2*x* and AA-2*x*; (ii) intra-cytotype outcrosses within each of AM-4*x*, AM-2*x* and AA-2*x*; (iii) intra-cytotype/inter-specific crosses between AM-2*x* and AA-2*x*; (iv) inter-cytotype/intra-specific crosses between AM-4*x* and AM-2*x*; and (v) inter-cytotype/inter-specific crosses between AM-4*x* and AA-

2*x*. The pollen-pistil interaction phase includes recognition and match between pollen and receptive stigma, pollen germination, tube growth down the style and into the ovary, and ovule penetration (Chapter 4; NGHIEM *et al.* 2012).

Pollen tubes grew well in the style during 24 h, entered into the ovary and penetrated the ovules within 72 h for all five mating types. However, there were differences in pollen-pistil interactions and subsequent seed yields amongst types of mating (Fig. 6.1).

6.5.1. Self- and outcross-mating within each of AM-2*x*, AM-4*x* and AA-2*x*

No significant difference in ovule penetration between selfs and outcrosses of AM-2*x* and AA-2*x* was detected; however, the self-pollinated flowers set no pods whereas on average 7 % of outcrossed flowers produced pods, and over 80 % of seeds in the pods were filled. It appears that variation in mature pod and seed yields between selfs and outcrosses of AM-2*x* and AA-2*x* was attributable to post-zygotic factors rather than pollen-pistil interactions. In contrast, AM-4*x*-selfed pollen tubes were more targeted towards the ovules than were the AM-4*x* outcross and 0.08 % of the AM-4*x* selfed flowers produced pods. Selfed seed numbers (mean of 3.7 total seeds/ pod) were similar to the mean yield of AM-4*x* OP pods (~ 4 seeds/ pod) (NGHIEM *et al.* 2011). This was expected as GRIFFIN *et al.* (2012) reported that approximately 98 % of progeny from six AM-4*x* clones were selfed. No pods were obtained from AM-4*x* outcrosses in this experiment given the limited number of pollinated flowers left after sampling for pollen tube growth observation, but in the following year I was able to obtain a small number of AM-4*x* outcross pods from a larger set of crosses.

6.5.2. Reciprocal crosses of AM-2*x* and AA-2*x*

Pollen tubes grew well in both AM-2*x* and AA-2*x* styles and penetrated ovules; however, the growth of AM-2*x*-pollen tubes in AA-2*x* styles was more oriented and more AA-2*x* ovules were penetrated than in the reciprocal combination. Better performance of *A. auriculiformis* as a maternal parent was also observed by SEDGLEY *et al.* (1992c). No pods were obtained in AM-2*x* x AA-2*x* crosses due to the limited number of flowers pollinated, while 8.5 % of pollinated flowers produced pods in AA-2*x* x AM-2*x* crosses, with a high ratio of filled seeds per pod.

6.5.3. Inter-cytotype crosses within and between the two species

Inter-cytotype crosses between species had a significantly greater number of ovules penetrated than the intra-species crosses. The direction of cross did not affect the number of pollen tubes per style and number of ovules penetrated in intra-species crosses. This indicated no unilateral incompatibility in the reciprocal of 2*x* x 4*x* within AM although the 2*x* styles were significant longer than the 4*x* styles (NGHIEM *et al.* 2011). In *Leucaena* species (*Leguminosae*) SORENSSON and BREWBAKER (1994) found a strongly unilateral incompatibility from 12 combinations out of 21 of reciprocal 2*x* x 4*x* of *L. diversifolia*, associated with unequal length of parents' styles. For inter-species crosses, AA-2*x* as a female presented more penetrated ovules per ovary than did AM-4*x*. However, yields of pods and filled seeds from all these inter-cytotype crosses were extremely poor, compared with those from the intra-cytotype crosses despite the demonstrated absence of pre-zygotic isolation. Thus, it appeared that factors operating between fertilization and seed maturation were involved in limiting the production of viable progeny from any cross involving gametes of 4*x* plants.

6.6. Pod and seed development from fertilization to maturity

An experiment was conducted to obtain quantitative and temporal information on pod and seed development following control-pollinated (CP) inter-cytotype crosses and the AM-4*x* outcross. Results were compared with observations of OP pod and seed development on each taxon. Intra-cytotype 2*x* crosses (Chapter 4) were not repeated but contributed insights to this stage of the reproductive process (Chapter 5).

6.6.1. Spike/ pod retention and pod set number per spike over time

Rates of abortion of spikes and pods following CP and OP were observed at 2, 3, 5, and 7 weeks after pollination (WAP) and at harvest (18 WAP) (Fig. 6.1). Generally, more OP spikes were retained than CP spikes initially (2 to 7 WAP) suggesting that impacts of bagging and handing in CP affected the spike retention; however, this did not affect the pod set (%) which was higher for CP in all cases. The spike retention reduced by about 50% at 7 WAP for both CP and OP with AM-2*x* and AA-2*x* mothers, while the majority of AM-4*x* spikes (both OP and CP) had dropped off by 2 WAP with a further threefold reduction by 7 WAP (Fig. 6.1). Thus, spike abortion began in the early post-fertilization period and was influenced by mating type.

Spike and pod abortion is typically a response to restricted availability of resources (water and nutrients) and/or environmental factors, such as weather conditions and pest and/or disease attack (KENRICK 2003; STEPHENSON 1992; TYBIRK 1993). In this study, in a common environment, final rates of pod set were similar at harvest time (0.2 - 0.3 %) for OP AM-2*x*, AM-4*x*, and AA-2*x* (Fig. 6.1).

This accords with the conclusions of other authors that less than 1 % yield of pods from female flowers following OP is common in acacias (KENRICK 2003; MONCUR *et al.* 1991; TYBIRK 1993).

Two factors probably contributed to the high pod set percentage in the majority of CP cross types, both at initial stages of pod development and at harvest time. The proportions of flowers that were pollinated would have been higher than for OP, and the reduction in flower density of the CP thinned spikes might result in reduced competition for resources between developing pods. However, the CP pod set percentage varied amongst inter-cytotype crosses. In the first 2 – 3 weeks of pod development 4*x* x 2*x* crosses produced more pods than 2*x* x 4*x* crosses. This could be due to 4*x* pollen being less viable than 2*x* pollen, as suggested by both *in vitro* and *in vivo* evidence (NGHIEM *et al.* 2012; NGHIEM *et al.* 2011), reducing fertilization success. Subsequently, the pod set percentages for all inter-cytotype crosses reduced at 5 and 7 weeks and were variable at 18 WAP (range of 0 - 1.57 %), as genic imbalance mechanisms and also resource competition possibly played their roles. As a result, the mature pod harvests of these inter-cytotype crosses were far lower than that of the diploid crosses, which exceeded 7 % on average within and between species (Fig. 6.1).

AM-4*x* outcrossed CP flowers yielded very few pods, an average of 2.18 % at 2 WAP and reducing to 0.26 % at harvest time with differences among the four individual crosses (0 – 0.45 %). The non-targeted pollen tube growth in the ovaries and the low number of ovules penetrated per ovary of the AM-4*x* outcross (Fig. 6.1) suggest that pre-zygotic factors may have contributed to the very low pod yield. No

other published reports comparing the effects of self- and outcross-pollination on 4*x* pod production in the family *Leguminosae* were found.

Mean number of pods per retained spike changed among CP crosses and OP parents over time because of spike losses. The pod number per AM-2*x* spike was unchanged throughout the maturation period, while AM-4*x* spikes with below average numbers of pods per spike dropped off first, resulting in an increase in the average pod number per spike from 3 weeks onwards. In *A. auriculiformis*, mean OP pod number per spike reduced by half from 3.2 at 2 weeks to 1.7 at harvest (Chapter 5 and Fig. 6.1). These three different patterns are difficult to relate simply to theories of formation of resource-sinks, in which pod development attracts resources (STEPHENSON 1992).

There was a similar trend in inter-cytotype crosses as spikes were also abscised; the mean numbers of CP pods remaining on each spike was affected by the maternal taxon. In particular, AM-4*x* outcrosses displayed a low spike and pod retention (Table 5.2).

6.6.2. Ovule development after fertilization

Across all OP taxa and CP combinations, the mean number of developing ovules within a pod was similar (12.8 ovules/ pod) among the pods sampled at 2, 5, and 7 weeks. The small ovule size at 2 weeks and the slow initial growth to 5 weeks is consistent with a dormant period of the zygote that has reported in many angiosperms (RAGHAVAN 1986; SEDGLEY and GRIFFIN 1989) and acacias. For example, in natural (*A. mangium* x *A.auriculiformis*) hybrids the dormant period has been reported as 2 months (SORNSATHAPORNKUL and OWENS 1999b). The larger

ovules of AM-4*x*, relative to AM-2*x* (Fig. 5.4), are like polyad and stigma diameter (NGHIEM *et al.* 2011) an expression of gigantism in the tetraploid. Proportions of undeveloped ovules were significantly higher in healthy inter-cytotype cross pods than in OP pods at 7 weeks, suggesting progressive abortion of inter-cytotype ovules within pods during pod development, with timing of abortion affecting the size of undeveloped seeds through to maturation of pods.

6.6.3. Seed yield and abortion

The seed yield index (SYI) was higher for OP AM-2*x* and AA-2*x* (average 1-2 filled seeds) than for OP AM-4*x* (0.64 filled seeds). Although there are some genetic differences between the sibling embryos in the pod (Chapter 5), it appears that most combinations in diploid outcrosses are compatible, resulting in high proportion of filled to total seeds per OP pod from the diploids in my study, 7.6 filled/ 8.3 total and 5.1 filled/ 6.2 total for AM-2*x* and AA-2*x*, respectively. However, for OP AM-4*x* only about 63% of total seeds were filled (mean 2.9/ 4.6). The high proportion of unfilled seeds in OP AM-4*x* pods was probably explained by irregularities of chromosome pairing occurring in the sequence of mitoses and meiosis that produce the male and female gametes (KENRICK and KNOX 1982). Both genetic load and genic imbalance from abnormal chromosome pairing are probably operating between individual zygotes in a pod, affecting seed development. Genetic factors are thus more important than competition for resources in explaining the difference between intra- and inter-cytotype crosses. However, the reason for very few number of filled seed in CP AM-4*x* outcrosses pod (0.6/ 5.0) is not clear.

For CP the SYI of diploid crosses within and between species (40 – 79 filled seeds) was much higher than that of the inter-cytotype crosses (less than 1 filled

seed). The reasons for the differences can be inferred to be primarily genetic and associated with the changes in ploidy level since the maternal resources were available to all trees in the orchard. Very low developed seed set in the reciprocal 2*x* x 4*x* have been reported for many taxa, such as *Vitis*, *Citrus*, and *Berberis* (EBADI *et al.* 2010; ESEN and SOOST 1973; HEO *et al.* 2007; HIRAMATSU *et al.* 2003; SUN *et al.* 2011; WAKANA *et al.* 2002; WAKANA *et al.* 2003). The seed failure was attributable to defective endosperm development. The endosperm has a critical function in nutrition and regulation of embryo differentiation (GRINI *et al.* 2002). Following 2*x* x 4*x* and 4*x* x 2*x*, the respective genomic ratios of maternal to paternal genomes in the endosperm are 2:2 and 4:1, both of which deviate from the usual 2:1 ratio, which is required for normal endosperm development (LESTER and KANG 1998; MOFFETT 1956; RAMSEY and SCHEMSKE 1998; RANNEY 2006; STIFT *et al.* 2010). Therefore, the significant higher numbers of unfilled seeds within mature pods in the CP inter-cytotype than in the CP intra-cytotype 2*x* outcross and OP parents could be due to early degeneration or abnormality of endosperm development which resulted in either embryo starvation or irregular embryo transition. Note that this does not explain the poor seed yield and high seed abortion in AM-4*x* outcross where genomic ratio in the endosperm would be in balance (4:2) so we must also assume that there are direct cytogenetic effectsthat affect the probability of survival of any particular 4*x* zygote.

6.6.4. Seed weight and viability

Significant reductions in weight of the inter-cytotype and AM-4*x* outcross seeds compared to those of OP parent seeds were demonstrated. This strongly suggested that the embryo in the inter-cytotype crosses and AM-4*x* outcrosses might

be only partially developed rather than fully developed as was observed in healthy OP seeds.

Embryoless seeds occurred as a common phenomenon in inter-cytotype crosses of grape and grapefruit (ESEN and SOOST 1973; WAKANA *et al.* 2002), and was considered as a consequence of failed embryo differentiation at the globular stage to form two cotyledon primordia, which is very important for seed maturity. Moreover, variation in seed sizes within the cross-types in this study might result from differences in the timing of endosperm degeneration.

This imperfect development of seed from inter-cytotype crosses and AM-4*x* outcrosses could explain the failure of the seed to germinate or to survive beyond 10 days from sowing (Chapter 5).

6.6.5. Genotyping to confirm crossing success

DNA was extracted from non-germinated or dead seeds of inter-cytotype crosses and genotypes determined. This allowed me both to assign ploidy and to confirm whether the putative parents were correct. More than 85 % of the progeny were confirmed to have arisen from the target crosses. Most were identified as 3*x* progeny by the presence of three alleles per locus or a flow cytometry technique. However, 8 out of 69 progeny presented only two alleles at the studied loci although the allelic profiles were consistent with the correct crosses having been made. These 8 were assumed to be diploids rather than triploids. This production of diploid progeny from inter-cytotype crosses might be related to chromosome behaviour, which is a cytogenetically complex in polyploids because of the occurrence of multivalent pairing. The review by RAMSEY and SCHEMSKE (2002) identified a high

frequency of aneuploidy, including hypo- and hyper-euploids, in the gametes of neopolyploids so we might expect this to be occurring in the *Acacia* although it is not possible to say from the data collected in my study.

In conclusion, this thesis has contributed to the understanding of reproductive behaviour of two different cytotypes of *A. mangium* and diploid *A. auriculiformis*. I have identified post-zygotic reproductive barriers, probably resulting from chromosomal and genic imbalance in developing endosperm, embryos and seeds, as the main factor limiting the yield of 3*x* seeds in quantity via inter-cytotype mating. Although seed development is a problem, hybridization between 4*x* and 2*x* for the ultimate purpose of producing viable 3*x* offspring of these tropical *Acacia* species is possible. Since there were differences among the few cross-combinations of each cross-type studied, testing a larger number of individual cross combinations would strengthen the conclusions drawn and would increase the chance of success by identifying favorable combinations. The application of *in vitro* culture techniques to rescue embryos of target crosses would be a worthwhile endeavour. This has been done successfully with *A. nilotica* (GARG *et al.* 1996) and several other species (ALEZA *et al.* 2010; NAVARRO *et al.* 2003; VILORIA *et al.* 2005). The success of embryo rescue depends upon the developmental stage of the embryo as well as the nutrients and plant growth regulators provided by the culture medium, which would be species-specific. Therefore, a separate study on embryo rescue is recommended to support polyploid breeding programs for tropical *Acacia* species. My study provided some evidence that selfs were actually more successful than outcrosses in producing viable offspring from 4*x* parents. From the practical viewpoint this is an important

result which could affect the breeding strategy. It is therefore worth further study to confirm this finding and to understand the mechanisms responsible.

Figure 6.1 Floral characteristics and breeding system of AM-2*x*, AM-4*x*, and AA-2*x* in relation with reproductive isolation

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